CHARACTERISATION OF A NEGATIVE REGULATOR OF HYDROPHOBIC AMINO ACID TRANSPORT IN *SACCHAROMYCES CEREVISIAE*

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LIST OF ABBREVIATIONS

A_{600}	absorbance at a given wavelength (eg 600 nm)
AAP	amino acid permease
ABC	ATP-binding cassette
ade	adenine
Ap^R	ampicillin resistance
BLAST	basic local alignment tool
bp	base pair
cfu	colony forming units
°C	degrees Celsius
CIAP	calf intestinal alkaline phosphatase
DCCD	dicyclohexylcarbodiimide
dH_2O	distilled water
EDTA	ethylenediaminetetra-acetic acid
EMSA	electrophoretic mobility shift assay
Fpa^R	yeast resistant to fluorophenylalanine (15 mg ml^{-1})
Fpa^S	yeast sensitive to fluorophenylalanine (15 mg ml^{-1})
GDHase	glutamate dehydrogenase
h	hour(s)
HECT	homologous to the E6-AP carboxyl terminus
his	histidine
HULA	histidine + uracil + leucine + adenine
HULAT	histidine + uracil + leucine + adenine + tryptophan
IPTG	isopropyl-β-thio-galactopyranoside
kb	kilobase
kDa	kilo-Dalton
leu	leucine
Lup^+	yeast capable of growing on limited leucine concentrations
Lup^-	yeast incapable of growing on limited leucine concentrations
M	molar
MAP	mitogen-activated protein
MFS	major facilitator superfamily
MIC	minimum inhibitory concentration

min	minute(s)
NCI	nitrogen catabolite inhibition
NCR	nitrogen catabolite repression
no.	number
NPR	nitrogen permease reactivation
n *x g*	n x acceleration force equivalent to Earth's gravity at sea level
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PGA	plate gradient assay
pfu	plaque forming units
pH	activity of hydronium ions at 25°C and 1 atmosphere pressure
PKA	protein kinase A
PMSF	phenylmethylsulfonylfluoride
RT	room temperature (approximately 25°C)
sec	second(s)
SDS	sodium dodecyl sulphate
Sm^R	streptomycin resistance
Tris	tris(hydroxymethyl)aminomethane
ts	temperature sensitive
Tc^R	tetracycline resistance
trp	tryptophan
UAS	upstream activating sequence
URS	upstream regulating sequence
ura	uracil
UV	ultraviolet
v/v	volume for volume
w/v	weight for volume
x-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

ABSTRACT

Yeast have many systems for nitrogen regulation. Combined, these systems make a complex network balancing uptake of nitrogenous compounds and their assimilation in diverse environments. Yeast preferentially use more easily assimilated nitrogen sources when they are available, and under these conditions, many permeases that transport less easily utilisable amino acids are inactive. This study characterises a spontaneous mutation that results in deregulation of an apparently unique hydrophobic amino acid permease (entitled Lup for Leucine uptake), in the presence of ammonium. The mutant, Lup^+ phenotype (ability to grow in leucine limiting conditions), was recessive and postulated to be due to a mutation in a gene, designated *LUP1*, which encodes a represser of the Lup permease. Since Lup^+ cells better accumulated hydrophobic amino acids, we used toxic amino acid analogs in growth media to select for *LUP1* (or *LUP1/lup1*) genotypes. Interestingly, we have observed that Lup^+ variants were more sensitive than their progenitor to not only the expected hydrophobic amino acid analogs L-methionine sulfoximine, L-ethionine and *m*-fluoro-D,L-phenylalanine, but also the nonhydrophobic analogs L-canavanine and L-azaserine. A screen of two wildtype yeast genomic libraries has identified 14 plasmids that complement the *m*-fluoro-D,L-phenylalanine sensitive (Fpa^S) phenotype of the *lup1* allele. Sequence data of complementing plasmids, extending outwards from transposons whose insertions defined the physical size of the complementing unit, has revealed that two genes, *BUL1* and *ARO4*, can complement the Fpa^S phenotype. *BUL1*, but not *ARO4*, could also render Lup^+ cells Lup^- (unable to grow in limited leucine environments), thus was predicted to be allelic to *LUP1*. Several additional lines of evidence demonstrated that *LUP1* and *BUL1* were allelic: (i) partial deletion of chromosomally encoded *BUL1* resulted in Lup^+ phenotypes; (ii) like *bul1* mutants, *lup1* mutants were temperature sensitive; (iii) *LUP1* and *BUL1* were in the same, or extremely close, chromosomal position; and (iv) Lup^+ mutants had an altered *BUL1* sequence to wildtype. The nature of changes to the *BUL1* sequence occurring in two Lup^+ variants consisted of point mutations occurring at different positions.

Bul1 is thought to be involved in the ubiquitination pathway due to physical interaction with the Rsp5 ubiquitin ligase. Rsp5 has been implicated directly with the ubiquitin-

dependent internalization and down-regulation of at least four yeast plasma membrane proteins. Rsp5 may also be involved in regulation of the Lup permease as a mutation in Lup1/Bul1 that eliminates its ability to bind to Rsp5 also abolishes its capacity to complement the Lup^{+} and Fpa^{S} phenotypes. Based on these phenotypes, the following model was proposed: Lup1/Bul1 functions with Rsp5 as an E3 complex for the recognition and subsequent ubiquitination of the Lup permease, targeting this protein for destruction in the presence of ammonium.

1. INTRODUCTION

1.1 NITROGEN NUTRITION

All organisms require nitrogen. It is an essential component of amino acids, proteins, purines, pyrimidines, nucleic acids, various vitamins and glucosamine. Elaborate systems that regulate and integrate nitrogen compound metabolism are thus required to ensure a constant nitrogen supply in the face of changing external and internal conditions. Control of nitrogen compound metabolism can be mediated through the induction and repression of enzyme systems responsible for nitrogen compound catabolism and degradation, and the positive and negative regulation of nitrogenous nutrient entry into the cell via permeases.

Amino acids comprise an important nitrogen source for *Saccharomyces cerevisiae* and given the critical role of nitrogen, it is not surprising that yeast have multiple and diverse permeases for the accumulation of amino acids over a wide range of physiological conditions. The various amino acid permeases in yeast differ with respect to substrate specificity, affinity, and velocity of transport. Permeases also differ in the mechanisms by which they are regulated, and the conditions in which they are active, particularly the nitrogen source present. This study investigates the negative regulation in the presence of ammonium, of a high affinity, hydrophobic amino acid transport permease in *S. cerevisiae*.

1.1.1 Assimilation of Primary Nitrogenous Nutrients

In order to fully comprehend the processes involved in amino acid transport, an understanding of nitrogen compound utilisation is required. More comprehensive treatises of yeast nitrogen metabolism and catabolism, and the regulation of these processes, are provided by Garraway and Evans (1984), and Wiame et al. (1985).

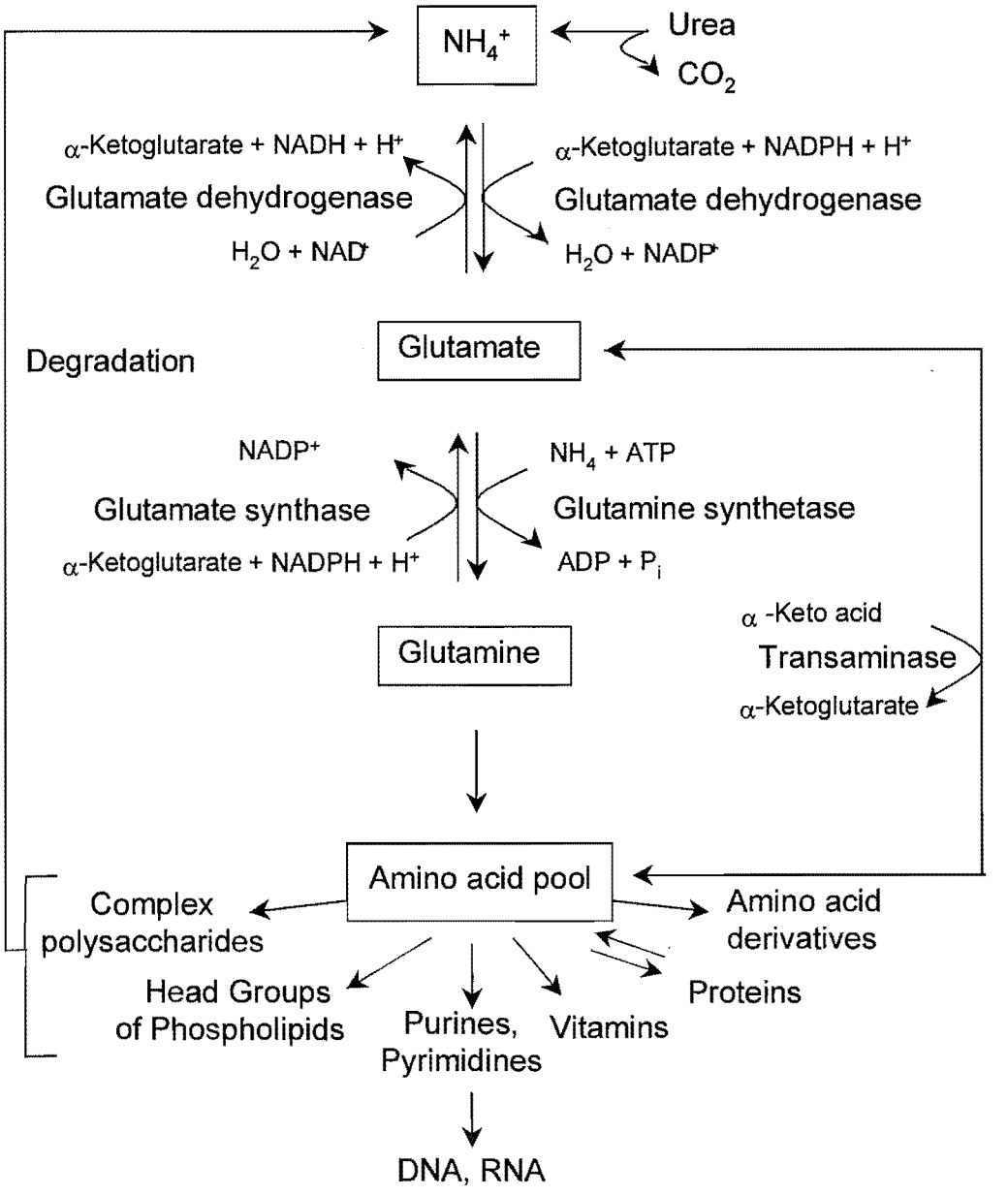

Figure 1.1: Overview of nitrogen utilisation in yeast, emphasising the central role of glutamine and glutamate as key metabolites.

Nitrogen sources that support the fastest growth rates in *S. cerevisiae* are ammonium ions, glutamine, glutamate and asparagine. These compounds are preferentially utilised by yeast, however, when they are not available or become limiting, a number of secondary nitrogenous compounds, such as most amino acids, proteins, allantoin and urea, can be used (Marzluf, 1997). Unlike other yeasts, *S. cerevisiae* cannot use nitrate or nitrite as the sole nitrogen source (Grenson, 1992).

Ammonia is assimilated into glutamate and glutamine, which are specifically needed for protein synthesis and as nitrogen donors for the synthesis of many other compounds (Figure 1.1). Degradation of nitrogenous compounds also yields glutamate and/or ammonium. The central metabolic role of glutamate was demonstrated in a study whereby glutamate comprised almost half the amino acid pool of cultures growing in a chemostat under conditions where glutamate was the limiting nitrogen source (Watson, 1976). When any one of 19 different amino acids was used as the sole nitrogen source, glutamate was the major constituent of the amino acid pool, followed by the nitrogen source added. Not surprisingly, the central metabolites glutamine and glutamate are considered the key substrates in regulation of nitrogen metabolism in yeast, as well as in other fungi and prokaryotes.

The initial step of ammonia assimilation involves the reductive amination of α-ketoglutarate, forming glutamate. The reversible reaction is catalysed by glutamate dehydrogenase (GDHase) (Figure 1.1). Two GDHases are present in *S. cerevisiae*, one specific for the NAD^+ and the other for the $NADP^+$ co-factor. The NAD^+-requiring enzyme could have a catabolic function because its activity is low when yeast are provided with ammonium or ammonium and glutamate, and its activity is higher when yeast are grown with glutamate only. Mutations in the structural gene for the $NADP^+$-GDHase (*gdhA*) demonstrate its anabolic function. The generation time of *gdhA*$^-$ mutants was doubled in media containing ammonium. However, it returned to normal when glutamate was also added to media (Wiame et al., 1985).

Three additional reactions play vital roles in the assimilation of primary nitrogen sources in *S. cerevisiae* (Figure 1.1). Glutamate is also produced from ammonia and α–ketoglutarate in a reversible reaction similar to that catalysed by GDHase, requiring the enzyme glutamate synthase and cofactor NADPH. Transamination, the reversible

transfer of the amino group from glutamate to an α-keto acid, is catalysed by transaminase, and yields the respective amino acid and α-ketoglutarate. Glutamate can also react with ammonia and ATP in the presence of glutamine synthetase to form the key metabolite, glutamine.

1.2 AMINO ACID TRANSPORT SYSTEMS

The outermost layer of the yeast cell envelope is the cell wall, through which pass freely solutes less than 600 Da in size. The yeast cell membrane provides the essential function of separating cellular reactants from the environment and refreshes intracellular supplies through selective permeability. The hydrophobic properties of the lipid bilayer allow passive diffusion of some lipophilic compounds such as fatty acids, alkanols and hydrocarbons (van der Rest et al., 1995), as well as free ammonia, water and oxygen. Transport of larger and/or more hydrophilic compounds, such as ammonium ions and amino acids, relies on protein permeases for transport. In many environments, these nutrients are in relatively low concentrations, thus require a process for accumulation against a concentration gradient.

1.2.1 Mechanisms of Amino Acid Transport

Amino acid permeases comprise the second largest family of transporters (the AAP family) behind sugar permeases, within the major facilitator superfamily (MFS) (Nelissen et al., 1995). Permeases belonging to MFS, also called the uniporter-symporter-antiporter family, are ubiquitous within all organisms, and along with the ATP-binding cassette (ABC) superfamily, account for nearly half of all known solute transporters in microorganisms. MFS transporters are classified as single polypeptide secondary carriers, capable of transporting small solutes. Transport is driven by chemiosmotic gradients, and MFS transporters lack any recognisable ATP-binding site. Along with amino acids and sugars, MFS permeases uptake or efflux a wide range of substances including drugs, an array of metabolites, nucleosides, vitamins, and inorganic and organic anions and cations (Pao, et al., 1998). The permeases have a

common structural topology with 10 to 14, but usually 12, transmembrane-spanning domains, commonly split into 2 structural units of 6 transmembrane-spanning domains often connected by a large cytoplasmic loop (Nelissen, et al., 1995). MFS members share a conserved amino acid sequence motif (RXGRR) between transmembrane-spanning domains 2 and 3, and a second similar, but less conserved, motif between domains 8 and 9 (Henderson and Maiden, 1990; Jessen-Marshall et al., 1995).

Transport of amino acids by yeast permeases occurs unidirectionally. Initially, in what may be the rate-limiting step, the amino acid to be transported is recognised and bound by a proteinaceous component of the yeast membrane relatively specific to that amino acid. It is unclear whether this component comprises the permease itself, or a separate, periplasmic, binding protein (Eddy, 1982; Kotliar and Ramos, 1983; Wainer et al., 1988). If a separate binding protein is involved, the amino acid is then transferred to the permease. Once bound, the permease binding site undergoes a reversible conformational change, resulting in translocation of the amino acid through the membrane, a process that requires energetic interactions involving coupling to a protonmotive-force-generating system. Finally, the amino acid is released into the cytoplasm.

The energy coupling mechanisms in yeast amino acid transport are consistent with the proton-dependent chemiosmotic coupling process, proposed by Mitchell (1963) to explain energy transduction in mitochondria, chloroplasts and bacterial membranes, and later used to describe active transport of nutrients. Protons are actively pumped out of the cell by plasma membrane proton ATPases coupled to ATP hydrolysis, generating an electrochemical gradient for protons, with a lower pH outside the membrane than inside. Protons have a thermodynamic tendency to flow back into the cell to equalise the pH, and amino acid translocation through the membrane is driven by the spontaneous influx of protons through the proton/amino acid symport system (Figure 1.2). Many researchers have documented the outflow of K^+ coupled to the influx of a stoichiometric number of protons (Horák, 1986; Eddy and Hopkins, 1989). The outflow of K^+ is considered non-essential for amino acid transport and is thought to be required simply to neutralise inflow of protons, particularly when the proton pump is not working, perhaps depending on energy metabolism (Eddy, 1982; Horák, 1986).

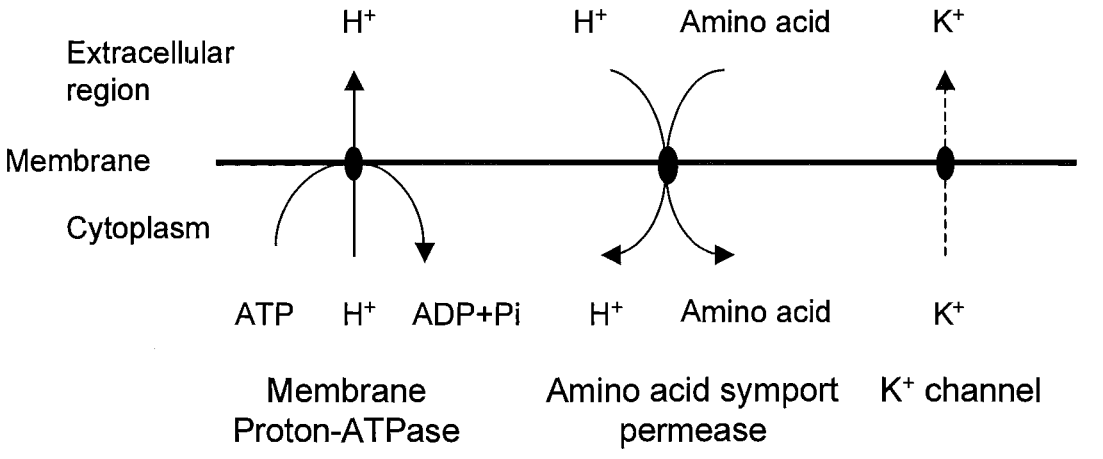

Figure 1.2: Scheme for amino acid membrane transport systems in yeast. Protons are pumped out of the cell via a membrane proton ATPase, requiring ATP hydrolysis, creating a proton gradient across the membrane. Amino acids are transported into the cell through amino acid symport systems, driven by the flow of protons back into the cell. Potassium ions may then flow out of the cell to neutralise the effects of protons flowing back into the cell.

The chemical and/or physical factors which affect energy production also influence amino acid uptake, suggesting that energy is required to transport amino acids. Kotyk and Rihova (1972a, b) have shown that uptake of glycine and γ-aminoisobutyric acid by *S. cerevisiae* increased when cells were preincubated with metabolizable carbon sources, and this coincided with an increase in the intracellular supply of high-energy phosphates. Similarly, Ramos et al. (1977) have seen increased uptake of [^{14}C]-labelled leucine after preincubation of starved *S. ellipsoideus* with D-glucose. Increases in preincubation time correlated with increased energisation of amino acid uptake. Propionaldehyde, a substrate that is capable of activating electron transfer and oxidative phosphorylation, also stimulated leucine accumulation. Inhibitors of energy production decreased or eliminated amino acid transport. Ramos et al. (1977) demonstrated that 2,4-dinitrophenol, which accelerates leakage of protons across the membrane, decreased leucine transport, as did antimycin, a specific inhibitor of mitochondrial electron transfer coupled to phosphorylation. Many other inhibitors, such as the glycolysis inhibitor indo acetate, the uncouplers azide, pentachlorophenol, and nystatin, ATPase inhibitors quercetin, dicyclohexylcarbodiimide, and diethylstilbestrol, and anaerobic conditions were all shown to inhibit amino acid uptake by *S. cerevisiae* (Ramos et al., 1975; Ramos et al., 1980; Kotyk and Dvoráková, 1990). Caution must be taken when

interpreting these results, however, as Horák et al. (1978) observed that some metabolic inhibitors at low concentrations could stimulate amino acid transport when cells were preincubated with glucose or ethanol. The researchers proposed that increased uptake was due to decreased degradation of amino acid permeases under these conditions.

One of the most important advances in understanding how amino acid transport is energised has been the demonstration of the intimate association between amino acid uptake with the uptake and efflux of protons, and the requirement of ATP. Several researchers have studied energisation of amino acid transport *in vitro* using purified proton-ATPase-containing yeast vacuolar or plasma membrane vesicles, rather than whole cells in order to eliminate the effects of cellular metabolism. Using right-side-out vacuolar vesicles, Ohsumi and Anraku (1981) have shown that [^{14}C]arginine was accumulated in the presence of ATP, but not ADP, AMP-adenosyl-5'-yl imidodiphosphate, NADH, glucose, succinate or lactate. Transport activity required the magnesium ion, and was sensitive to the vacuolar membrane ATPase inhibitor dicyclohexylcarbodiimide (DCCD), suggesting that a Mg^{2+}-requiring, DCCD-sensitive vacuolar membrane proton-ATPase was involved. The ATP-dependent formation of a pH gradient across the membrane during solute uptake was monitored by measuring fluorescent changes of the voltage-sensitive dye, 9-aminoacridine, during solute uptake. These changes were reversed in the presence of the uncouplers SF6847 or nigericin. Using the same system, Sato et al. (1984) have extended the evidence of the involvement of an ATP-dependent, proton/amino acid transport mechanism for the vacuolar membrane transport of 10 amino acids. Eddy and Hopkins (1986) have used yeast suspensions, in which energy metabolism has been inhibited artificially by the addition of antimycin, preventing ejection of protons through the proton pump. The pH gradient created prior to inhibition of proton efflux was sufficient to drive the transport of a small amount of amino acid, and using a pH electrode and K^+-selective electrode, Eddy and Hopkins have monitored the changes in pH and K^+ associated with [^{14}C]glycine uptake. They found a stoichiometric relationship of about two protons absorbed and two K^+ ejected per amino acid equivalent. The stoichiometry varies from one to two, depending on the amino acid symport assayed (Eddy, 1982; Horák, 1986; Eddy and Hopkins, 1989).

The role of conserved residues within yeast amino acid permeases, in proton and amino acid binding, and the exact mechanism by which protons and amino acids are translocated across the membrane, remains to be elucidated.

1.2.2 Yeast Have Multiple Amino Acid Transporters

Over 20 amino acid transport systems have been characterised genetically from transport mutants and/ or from kinetic analyses in *S. cerevisiae* (Table 1.1). Those that have been described genetically are between 30 and 65% similar at the amino acid level (excluding Mup1 and Mup3; Isnard et al., 1996), suggesting a possible common ancestor (Vandenbol et al., 1989; Janiaux and Grenson, 1990; Grenson, 1992; Goffeau et al., 1993; André, 1995; Nelissen et al., 1995). Several other putative permease genes have been identified by a search for similar open reading frames (ORFs) within the entire yeast genome (Goffeau et al., 1993; Nelissen et al., 1995). The products of two of these genes transport glutamine and asparagine (*AGP1*) (Schreve et al., 1988), and branched chain amino acids (*BAP3*) (Mai and Lipp, 1994; Didion et al., 1998).

Permeases differ in their substrate specificity, substrate affinity, regulation, and capacity. The wide variety of permeases for amino acid transport illustrate the precision by which the yeast cell can operate, accumulating amino acids at differing rates over a vast range of external conditions. The broad specificity, large capacity, and regulation according to nitrogen availability make the Gap1 permease, for example, well suited for taking up amino acids as a nitrogen source for a catabolic role, although there is no exclusive utilisation of a specific permease for a particular purpose. In nutrient-rich environments, yeast can afford to be more selective, and individual, usually constitutively expressed, permeases are the main transporters, best suited for providing amino acids to be incorporated into proteins. High affinity transporters allow yeast to scavenge amino acids, even at trace concentrations, while transporters with low affinity can transport amino acids when they are plentiful.

Table 1.1: Amino acid uptake systems identified in *S. cerevisiae*. Unless specified otherwise, L-amino acids are transported by permeases. Positions of ORFs that encode the various permeases were acquired from The Yeast Proteome Database (http://www.proteome.com/databases/YPD/YPDsearch-quick.html; Costanzo et al., 2000).

Transport System	ORF No.	Amino Acid Specificity	References
Gap1 general amino acid permease	YKR039W	all naturally occurring amino acids, many D-amino acids and several toxic analogs	Grenson et al., 1970 Jauniaux and Grenson, 1990
Can1 arginine permease	YEL063C	arginine, canavanine, lysine, histidine	Grenson et al., 1966 Grenson, 1966 Hoffmann, 1985
Lyp1 high affinity lysine permease	YNL268W	lysine	Grenson, 1966 García and Kotyk, 1988 Sychrova and Chevallier, 1993
Met-p_1/Mup1 high affinity methionine permease	YGR055W	methionine	Gits and Grenson, 1967 Isnard et al., 1996
Hip1 histidine permease	YGR191W	histidine	Tanaka and Fink, 1985
Put4 proline-iminoacid permease	YOR348C	proline, GABA	Lasko and Brandriss, 1981 Jauniaux et al., 1987 Vandenbol et al., 1989
Agp1/Wap1 broad specificity amino acid permease	YCL025C	leucine, isoleucine, valine, threonine, phenylalanine, tyrosine, serine, methionine, alanine, glutamine, histidine, asparagine, glycine	Grenson and Dubois, 1982 Schreve et al., 1998 Iraqui et al., 1999a
Gnp2 glutamine permease		glutamate, aspartate, alanine	Grenson and Dubois, 1982
Bap2/S1 branched-chain amino acid permease	YBR168C	leucine, isoleucine, valine	Didion et al., 1996 Kotliar and Ramos, 1983 Schreve and Garrett, 1997 Grauslund et al., 1995
Bap3/Pap1 branched-chain amino acid permease	YDR046C	branched-chain amino acids	Mai and Lipp, 1994 Didion et al., 1998 de Boer et al., 1998

Table 1.1: continued

Transport System	ORF No.	Amino Acid Specificity	References
Tat1/Vap1 high affinity tyrosine permease	YBR069C	tyrosine, tryptophan, valine, histidine, leucine, isoleucine	Kotyk and Dvoráková, 1990 Schmidt et al., 1994 Bajmoczi et al., 1998
Uep/Dal5 ureidosuccinate-allantoate permease	YJR152W	allantoate, ureidosuccinate	Rai et al., 1987 Rai et al., 1988
Tat2/Scm2/Ltg3 tryptophan permease	YOL020W	tryptophan	Schmidt et al., 1994
S2 low affinity, high velocity leucine transport system		leucine	Kotliar and Ramos, 1983
Uga4 GABA-specific permease	YDL210W	GABA	André et al., 1993
met-p_2 low affinity methionine permease		methionine, leucine, histidine, threonine, phenylalanine	Gits and Grenson, 1967 Isnard et al., 1996
Dip5 high affinity, high capacity dicarboxylic amino acid permease	YPL265W	glutamate, aspartate	Regenberg et al., 1998
Gup1 constitutive glutamic acid permease		di-carboxylic amino acids eg glutamic acid	Darte and Grenson, 1975
Gup2 ammonium-sensitive glutamic acid permease		di-carboxylic amino acids eg glutamic acid	Darte and Grenson, 1975
Gnp1 high affinity glutamine permease	YDR508C	glutamine	Xhu et al., 1996
Mup3 very-low affinity methionine permease	YHL036W	methionine	Isnard et al., 1996
Lup leucine uptake permease		leucine, methionine, tryptophan , isoleucine, threonine, phenylalanine	Heinemann et al., 1994

1.2.3 Systems of Hydrophobic Amino Acid Transport

As this study is concerned with the regulation of a hydrophobic amino acid permease, discussion of specific permeases is restricted to those responsible for the transport of hydrophobic amino acids. In particular, systems described in the subsequent sections include the permeases with wide specificity, Gap1 and Agp1, as well as those more specific for transport of leucine, methionine and tryptophan. Included under the umbrella of leucine, methionine and tryptophan-specific permeases are those permeases that are also specific for other hydrophobic amino acids such as phenylalanine, isoleucine and valine. Amino acids and toxic amino acid analogs referred to in this text are in the L-configuration, unless specified otherwise.

1.2.3.1 Gap1 Permease

Grenson et al. (1970) originally predicted that yeast had a nitrogen repressible, general amino acid permease to explain the phenomenon whereby cells, deficient in specific permeases for arginine, lysine and methionine, were resistant to the corresponding toxic amino acid analogs canavanine, thiosine and ethionine in the presence of ammonium, but were sensitive in the presence of proline. The rate of uptake of a number of other, considerably structurally different, amino acids, including glutamine, methionine, glycine, glutamate, tryptophan, alanine, aspartate, and citrulline, were shown to be increased in the presence of proline (Grenson et al., 1970; Cooper, 1982). Studies assessing the inhibition of [^{14}C]-labelled arginine, tryptophan, lysine and citrulline uptake by nonlabelled varieties of these amino acids, were carried out in the presence of proline, in a strain with mutations in the genes for specific arginine and lysine permeases. Lineweaver-Burk plots indicated that substrate uptake was competitively inhibited in each pairwise inhibition assay. The same carrier therefore transported all four amino acids. Presumptive evidence for a broad specificity amino acid permease was also shown using an arginine permease-less strain (*can1*). The *can1* variant was released from canavanine sensitivity when a wide range of amino acids and related compounds were present, presumably because the other amino acids were competitive inhibitiors of canavanine uptake, although the experiment does not reveal the mechanism of inhibition. Darte and Grenson (1975) have demonstrated that the substrate specificity of the permease, designated Gap1 (General amino acid permease) also includes acidic amino acids. While it was generally accepted that proline, an imino

acid, was not transported by Gap1 (Grenson et al., 1970), Lasko and Brandriss (1981) have demonstrated decreased proline uptake by *gap1* mutants compared with wildtype. Therefore, low affinity transport of proline may be mediated by the Gap1 system. Grenson et al. (1970) have isolated a *gap1* mutant in a *can1* strain resistant to canavanine, ethionine and thiosine in the presence of proline. Rytka (1975) has also isolated a *gap1* mutant by selecting for resistance to D-amino acids. These mutations that abolish Gap1 activity occur in the same gene, and Janiaux and Grenson (1990) have cloned and sequenced this *GAP1* gene.

Kinetic studies of leucine uptake by wildtype cells grown in the presence of proline demonstrated the existence of a leucine-transporting system with a high V_{max} that is partially inhibited (34-47%) by citrulline. Citrulline had no effect on [^{14}C]leucine transport by *gap1* mutants or cells grown on media containing ammonium as the nitrogen source. Under these conditions, the very high V_{max} was also no longer observed (Kotliar et al., 1994).

1.2.3.2 Specific Leucine Transporters

Kinetic studies of [^{14}C]leucine uptake carried out by Ramos et al. (1980) and Kotliar and Ramos (1983) consist of at least two Michaelis-Menten-obeying systems, designated S1 and S2. S1 is a high affinity and low velocity transport system whereas S2 is a low affinity, high velocity system. As these studies were carried out using yeast that had been grown on rich media containing a complex nitrogen source, the involvement of the Gap1 system could not be eliminated. Kinetic studies carried out in conditions that suppress Gap1, or *gap1* mutants, also revealed the presence of two leucine transport systems, the activities of which were not inhibited by D-leucine, D-isoleucine or D-valine. Kinetic parameters of these systems included K_m values similar to, but V_{max} values lower than, K_m and V_{max} values reported for S1 and S2. V_{max} values were substantially lower when cells were grown in media containing ammonium as a sole nitrogen source compared with proline, indicating that S1 and S2 are also sensitive to ammonium repression (Kotliar et al., 1994).

By selecting genes that suppress an isoleucine auxotrophy when overexpressed, Grauslund et al. (1995) cloned a putative isoleucine permease gene, similar to known yeast amino acid permease genes. Deletion of this gene in a *gap1* background reduced

[^{14}C]-labelled leucine and valine uptake by about 45% and isoleucine uptake by 25%. The uptake of other hydrophobic amino acids including alanine, phenylalanine, and methionine were not affected, however. These results suggested that the cloned gene encoded a permease with high amino acid specificity, responsible for a substantial amount of leucine, isoleucine and valine transport, thus the gene was named *BAP2* (Branched-chain Amino acid Permease). *BAP2* was later independently isolated by Schreve and Garrett (1997) as a multicopy suppressor of the YPD$^-$ phenotype (unable to grow on rich medium) of *aat1 leu2* yeast. *aat1* cells were unable to grow on rich media when auxotrophic for leucine, whereas *aat1 LEU2* mutants grew normally. A comparison of leucine uptake kinetic analyses between wildtype and yeast strains in which *BAP2* was disrupted showed that *BAP2* encoded the high affinity S1 leucine permease. K_m values obtained using wildtype cells grown in minimal ammonium medium, but absent from *bap2* variants, were very similar to those reported by Kotliar et al. (1994), consisting of 0.037 and 0.04 mM, respectively. By selecting for mutants resistant to the toxic leucine analog trifluoroleucine, Chianelli et al. (1996) have also isolated a mutation, *let1*, in which the S1 system was completely inactivated. *LET1* may therefore be an allele of *BAP2*.

Strains deleted for the *BAP2* gene have enabled better characterisation of permeases with lower leucine affinities. Data of leucine transport by a *bap2 gap1* strain, or a *bap2* strain grown in Gap1-repressing conditions, yields a non-linear Eadie-Hofstee plot, suggesting the presence of not one, but two additional leucine transport pathways (Schreve and Garrett, 1997). Unk1 has a lower affinity, but higher capacity of leucine transport than Bap2, thus is predicted to be equivalent to S2. K_m values for leucine uptake via Unk1 (0.58 mM) are higher than those reported for uptake by S2 (0.14 mM) (Kotliar et al., 1994), although this discrepancy is likely to be due to the contribution to leucine uptake by other permeases, in the latter results. Schreve and Garrett labelled the third leucine transport pathway Unk2. Unk2 has a very low substrate affinity and velocity, and thus, contributes little to leucine transport. Trifluoroleucine resistant strains isolated by Chianelli et al. (1996), contained a mutation in *LET1* (*BAP2*), and also a second gene, designated *LET2*. Mutations in *LET2* resulted in inhibition of S2, significantly lowering the V_{max} of this system. Possible ways that S2 was inhibited include decreased delivery of S2 to the membrane, or decreased ability of the S2 permease to transport an amino acid, without affecting its affinity. Together, *let1* and

let2 mutations affected uptake of leucine, valine, isoleucine, methionine, threonine, serine and phenylalanine. Moreover, these amino acids, as well as cysteine, alanine, serine and threonine, but not lysine, arginine or proline, inhibited leucine uptake by a *let1 let2 gap1* strain. The S2 system therefore appears to have a fairly wide, but not unlimited substrate specificity, transporting hydrophobic and hydroxy amino acids.

Systematic sequencing of the yeast genome has revealed an ORF, *PAP1* (Putative Amino Acid Permease; Mai and Lipp, 1994), that is similar to amino acid permease genes. *PAP1* is most similar to *BAP2*, thus was renamed *BAP3*. Like the Bap2 permease, the *PAP1* gene product is responsible for the transport of leucine and other branched-chain amino acids (Didion et al., 1998). *BAP3* is a potential candidate for encoding the S2 permease. A comparison between kinetic parameters for transport by the two systems would be useful to investigate whether they are indeed identical.

1.2.3.3 Specific Methionine Transporters

Kinetic studies of [^{14}C]methionine uptake suggested the presence of at least two methionine transporters. One system, met-p_1, has a high affinity for methionine (K_m of 12 μM). Yeast deficient in this system have been isolated by their resistance to low doses of ethionine. Mutants were not resistant to low doses of canavanine, thiosine, β-2-thienylalanine, or D,L-*p*-fluorophenylalanine, thus this system has a high substrate specificity. The high specificity was also demonstrated by a lack of competition to methionine uptake in the wildtype by a wide range of amino acids. Uptake was only inhibited by the methionine analogs D-methionine, ethionine and D,L-selenomethionine (Gits and Grenson, 1967). Isnard et al. (1996) have also isolated mutants in this high affinity methionine permease, by selecting for resistance to methionine sulphoxide and ethionine sulphoxide, and they have cloned the permease locus, designated *MUP1* (Methionine Uptake).

In the same way that Bap2 leucine permease mutants have been useful for the study of lower affinity leucine permeases, high affinity methionine permease mutants have enabled the characterisation of lower affinity methionine permeases. Using met-p_1 mutants, Gits and Grenson (1967) established that the K_m of the low affinity methionine permease, met-p_2, was 0.77 mM. Mutants were ethionine sensitive and could transport

methionine when grown in the presence of proline but not ammonium, suggesting that the met-p_2 system is subject to nitrogen catabolite repression. A kinetic study of methionine transport by *mup1* mutants divulged that the met-p_2 system was comprised of two systems; a low affinity methionine permease with a K_m of 0.2 mM, and a very low affinity system with a K_m of approximately 1 mM (Isnard et al., 1996). An ORF with a similar sequence to *MUP1*, designated *MUP3*, was found in the yeast genome database. Disruption of *MUP3* in a *mup1* strain eliminated the very low affinity methionine permease. Methionine transport by the *mup1 mup3* strain was inhibited by ethionine, leucine, histidine, threonine and phenylalanine (Isnard et al., 1996), extending the substrate specificity profile of the met-p_2 permease past methionine, ethionine, threonine, serine and D,L-selenomethionine (Gits and Grenson, 1967).

Mup1 and Mup3 contain 13 putative membrane-spanning domains, and do not share amino acid sequence domains that are highly conserved in all other *S. cerevisiae* amino acid permeases identified, suggesting that Mup1 and Mup3 define a new family of amino acid permeases.

1.2.3.4 Specific Tryptophan Transporters

Using a *can1 gap1* strain, Kotyk and Dvoráková (1990) have observed tryptophan uptake by a system with a K_m of 0.41 mM. This system appeared to have a broad specificity, as tryptophan uptake was strongly competitively inhibited by 100-fold higher concentrations of methionine, phenylalanine, tyrosine, serine, tryptophan, cysteine and arginine. Many remaining naturally occurring amino acids inhibited uptake to a lesser degree.

Import of tryptophan, histidine and leucine is blocked by the immunosuppressive drug FK506, so strains auxotrophic for these amino acids are sensitive to the drug. By selecting for clones that confer resistance to FK506, Schmidt et al. (1994) have isolated the genes *TAT1* and *TAT2*. Disruption of *TAT2* only conferred a growth defect when combined with the *trp1* allele causing tryptophan auxotrophy, thus researchers predicted that *TAT2* encoded the high affinity tryptophan permease. Indeed, the *tat2* mutant showed severely decreased levels of amino acid import of [^{14}C]-labelled tryptophan, but neither tyrosine nor histidine. *TAT1* disruption, however, prevented growth of tyrosine auxotrophs (contain the *tyr1* allele), and tyrosine uptake by these strains was abolished.

This gene was therefore predicted to encode a high-affinity tyrosine transporter. Tryptophan uptake was also decreased slightly in the *TAT1* mutant, and overexpression of *TAT1* could overcome the growth defect of *tat2 trp1* mutants, thus low affinity or low capacity uptake of tryptophan is also mediated by the Tat1 permease. By selecting for clones that conferred increased histidine transport, Bajmoczi et al. (1998) have demonstrated that *TAT1* also encodes the low affinity histidine transporter. In addition, During-Olsen et al. (1999) have provided evidence that this system transports cysteine. As tryptophan transport occurs by at least two pathways in addition to the Gap1 permease, kinetic data and amino acid inhibition profiles for tryptophan transport obtained by Kotyk and Dvoráková (1990) probably reflect a combination of systems. Whilst the Tat2 permease contributes to high affinity tryptophan uptake, the substrate specificity of this system is very narrow. The Tat1 permease appears to have a wider specificity, and could contribute more to the hypothetical broad specificity tryptophan system described by Kotyk and Dvoráková (1990).

1.2.3.2 Agp1 Permease

AGP1, encoding a wide specificity permease, was independently discovered by isolation of variants which were more sensitive than wildtype strains to toxic analogs of asparagine and glutamine (Schreve et al., 1998), and during systematic sequencing of chromosome III (Oliver et al., 1992). Under nitrogen-limiting conditions Agp1 transports primary substrates asparagine and glutamine with intermediate affinity, with K_m values of 0.29 mM and 0.79 mM, respectively (Schreve et al., 1998). Doubling times of *agp1* mutants were increased compared with the wildtype, when strains were grown in media containing high (millimolar) concentrations of leucine, isoleucine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine or valine. Therefore, Schreve et al. (1998) predicted that Agp1 also transports these amino acids, albeit with a lower affinity. Moreover, aspartic acid, glutamic acid, isoleucine, leucine, methionine, serine, and threonine significantly inhibited uptake of [^{14}C]-asparagine. Iraqui et al. (1999a) have demonstrated that expression of *AGP1* is induced by the addition of various amino acids to media. These researchers have investigated the substrate specificity of Agp1 by comparing the initial uptake of amino acids by *gap1* and *gap1 agp1* strains after induction by citrulline (citrulline does not interfere with amino acid transport via Agp1). In the *gap1* strain, Agp1 was responsible for a significant portion of initial uptake of many amino acids, including leucine (96%), isoleucine (86%),

tyrosine (83%), valine (82%), phenylalanine (78%), threonine (77%), methionine (68%), glutamine (64%), serine (62%), alanine (60%), histidine (54%), glycine (49%) and asparagine (25%). Agp1 contributed negligibly (0-15%) to the uptake of tryptophan, proline, arginine, aspartate, glutamate and lysine. Schreve et al. (1998) have suggested that the affinity of Agp1 is lower for amino acids other than the so-called primary substrates, asparagine and glutamine. However, data presented by Iraqui et al. (1999a) is inconsistent with this conclusion. K_m values for Agp1 transport of leucine, isoleucine and phenylalanine are 0.16, 0.6 and 0.6 mM, respectively. Thus affinity of Agp1 is relatively low and appears to be in same range for several amino acids. Interestingly, Iraqui et al. (1999a) did not find any major contribution by the Bap2 permease, the so-called major branched-chain amino acid permease, in the utilisation of leucine or isoleucine when these were provided at 1 mM concentrations, or the Tat1 permease in tyrosine accumulation when this amino acid was used at 1-10 mM. Agp1 is a good candidate for the low affinity methionine permease as both have a broad specificity range. Also, methionine transport mediated by this low affinity methionine permease is inhibited by leucine with an apparent K_i value of 0.3 mM (Isnard et al., 1996), which is very close to the K_m for leucine transport by Agp1, measured under similar growth conditions (Iraqui et al., 1999a).

1.3 REGULATION OF AMINO ACID UPTAKE BY NITROGEN SOURCE

The first point at which yeast exercise control over the use of nitrogenous compounds occurs at the plasma membrane. Control of amino acid transport therefore comprises a crucial aspect of regulation of nitrogen catabolism. Factors affecting amino acid transport are many-fold, and the effects on transport of factors affecting energy coupling and production have been discussed previously. The pH of the environment influences amino acid transport by determining the charge of the amino acid and carrier protein, and hence binding capacity, and an adequate supply of protons is essential for maintaining a proton gradient (Olivera et al., 1993). Temperature and the presence of other nutrients present in media, such as salts, also affect amino acid uptake rates (Norbeck and Blomberg, 1998). The present study has implicated the importance of, and is primarily concerned with, the regulation of amino acid transport systems by the nitrogen source present in media. Mechanisms of regulation of transport systems

described thus far are exerted at the level of permease production by induction or repression of gene expression, and at the post-translational level, inhibiting permease function and controlling permease turnover.

Many amino acid permeases are repressed when yeast are grown in readily utilisable nitrogen sources such as ammonium, glutamine, glutamate and asparagine, and derepressed when grown in poor nitrogen sources such as proline. Three processes responsible for this phenomenon have been identified, including nitrogen catabolite repression (NCR), nitrogen catabolite inhibition (NCI) and nitrogen permease reactivation (NPR). Several overlapping layers of control of permeases regulated by these processes enable a finely tuned response by the yeast cell to the nitrogen source quality of the environment. Permeases may also be regulated by the amino acids they transport. The synthesis of a number of amino acid permeases is induced by the presence of amino acids, thus permeases are synthesised at a low level when there are no amino acids to transport. Amino acids may also inhibit permease function, and possibly further synthesis, when sufficient quantities have been transported by the cell.

1.3.1 Nitrogen Catabolite Repression (NCR)

The physiological response whereby genes encoding permeases and enzymes for catabolism of poorly used nitrogen sources are repressed in the presence of readily utilisable nitrogen sources, and activated in their absence, is deemed nitrogen catabolite repression (NCR). NCR-sensitive permease genes include *GAP1* (Grenson et al., 1970), *GNP1/AGP1* (Schreve et al., 1998), *PUT4* (Lasko and Brandriss, 1981; Jauniaux et al., 1987; Vandenbol et al., 1989), *UEP/DAL5* (Rai et al., 1987), and *UGA4* (Wiame et al., 1985). NCR-sensitive genes encoding catabolic enzymes include the proline utilisation genes *PUT1* and *PUT2* (Marzluf, 1997), allantoin catabolic genes (Bossinger et al., 1974), genes for asparagine degradation (Dunlop et al., 1980), *GLN1* encoding glutamine synthase (Valenzuela et al., 1998), *GDH2* encoding the catabolic NAD-linked GDHase (Roon and Even, 1973), *GDH1* encoding NADP-linked GDHase (Nagasu and Hall, 1985), and the gene encoding arginase (Courchesne and Magasanik, 1988). An important source of nitrogen for yeast is intracellular protein, which undergoes rapid degradation under certain circumstances such as upon entry into

stationary phase or sporulation. Coffman and Cooper (1997) have reported that some, but not all, vacuolar protease genes are regulated by NCR. This global regulatory mechanism is also involved in induction of genes for pseudohyphal growth in response to severe nitrogen starvation, which might be a nitrogen scavenging mechanism (Lorenz and Heitman, 1998b).

It was originally thought that permease genes under NCR-control were expressed constitutively, with ammonium affecting the carrier allosterically (Grenson et al., 1970; Rytka, 1975). Northern analyses revealed that mRNA levels of *GAP1* (Jauniaux and Grenson, 1990), *PUT4* (Jauniaux et al., 1987) and *UEP/DAL5* (Rai et al., 1987) were strongly reduced when ammonium was the nitrogen source, compared with levels achieved when yeast were grown in proline or urea. These results clearly indicated that NCR-mediated permease regulation involved a control of transcript formation and/or stability.

1.3.1.1 Ure2

The first enzyme involved in the regulation of NCR-sensitive genes to be isolated was the product of the *GDHCR*, *URE2* or *USU* gene. The *ure2* mutation allowed growth in the presence of ammonium of a strain that contained mutations in the genes that encode NADP-GDHase and glutamate synthase (a glutamate auxotroph). Ure2 was predicted to be a negative regulator of NAD-GDHase in response to nitrogen source. NAD-GDHase was deregulated in the *ure2* mutant, enabling limited conversion of ammonia to glutamate by the reaction that NAD-GDHase normally catalyses in the reverse direction (Grenson et al., 1974). Mutations in *URE2* were subsequently shown to release a number of enzymes from ammonium repression, thus the negative regulatory function of Ure2 is pleiotropic (Drillen and Lacroute, 1972; Drillen et al., 1973; Grenson et al., 1974). Following the demonstration that NCR is exerted at the level of gene expression, the role of Ure2 as a negative represser of NCR-sensitive genes was shown. *GAP1* and *PUT4* mRNA levels from *ure2* variants cultured in the presence of ammonium were considerably higher than levels from the isogenic wildtype, although not as high as when strains were grown in a non-repressible nitrogen source such as proline. These results suggested that an additional negative regulator also controlled gene expression in response to nitrogen source (Jauniaux et al., 1987; Jauniaux and Grenson, 1990). Coschigano and Magasanik (1991) cloned *URE2*, and the predicted

amino acid sequence obtained was not similar to DNA-binding proteins, although resembled glutathione *S*-transferases.

1.3.1.2 Gln3

A second global regulator controlling NCR-sensitive gene expression, Gln3, was identified as a factor required for high-level production of glutamine synthetase activity (Mitchell and Magasanik, 1984). Researchers have subsequently demonstrated that Gln3 is required for high-level gene expression and/or production of activity of virtually all described NCR-sensitive permeases and enzymes (Courchesne and Magasanik, 1988; Daugherty et al., 1993; Coffman et al., 1995).

A *cis*-acting element upstream of *DAL5*, UAS_{NTR} (Upstream Activating Sequence), was found to be necessary and sufficient for Gln3-mediated activation and NCR-sensitive expression of *DAL5* (Cooper et al., 1989). Saturation mutagenesis of the UAS_{NTR} element (Bysani et al., 1991) identified a dodecanucleotide with the DNA sequence 5'-GATAA-3' at its core. This consensus sequence was subsequently identified upstream of all recognised NCR-sensitive genes (Daugherty et al., 1993; Marzluf, 1997). The Gln3 structure, predicted from *GLN3* sequence information, would contain, in a central region, a zinc-finger DNA-binding domain. The zinc-finger motif was similar to two zinc-fingers within the transcription factor, GATA-1, which bind to GATA consensus sequences in regulatory regions of erythroid cell-specific genes. It also resembled single zinc-finger proteins of the *Aspergillus nidulans* nitrogen regulatory factor, AreA, and the nitrogen regulatory transcription factor of *Neurospora crassa*, Nit2, which binds DNA (Minehart and Magasanik, 1991). Genes encoding similar GATA factors involved in nitrogen regulation have since been discovered in other fungi, including *NRE* of *Penicillium chrysogenum* and *NUT1* in *Magnaporte grisea* (Marzluf, 1997). GATA zinc-finger DNA binding domains consist of one or two tandem copies of a metal-binding domain encompassing the residues $Cys\text{-}X_2\text{-}Cys\text{-}X_{17}\text{-}Cys\text{-}X_2\text{-}Cys$, with a central loop of 17 amino acids, and an immediately adjacent basic region (Evans and Felsenfeld, 1989). Site-directed mutational and three-dimensional studies of conserved regions in various GATA factors revealed that this central loop contributed significantly to DNA binding affinity. A conserved leucine residue within this loop was important for recognition of promoter elements, and interacts with DNA within the major groove. Conserved tryptophan and arginine residues were responsible for maintaining the

structural integrity of the domain for zinc chelation (Marzluf, 1997). By site-directed point-mutational studies, Svetlov and Cooper (1997) have demonstrated that in addition to the zinc-finger motif, the minimal Gln3 domain required for mediating transcriptional activation consists of 13 acidic and hydrophobic amino acids which are predicted to form an α-helix. Immunoprecipitation experiments using a Gln3 polyclonal antibody have shown that Gln3 binds the UAS_{NTR} element of *GLN1* (Minehart and Magasanik, 1991), agreeing with gel shift and footprinting studies demonstrating binding of purified Gln3 to UAS_{NTR} sites upstream of *GDH2* and *GLN1* (Blinder and Magasanik, 1995). Overwhelming evidence therefore suggests that Gln3 positively activates transcription of NCR-sensitive genes by binding to UAS_{NTR} sites.

The *ure2* and *gln3* mutations result in opposing phenotypes. Courchesne and Magasanik (1988) have studied the effects of *ure2* and *gln3* single or double mutations on the expression of NAD-GDH, glutamine synthetase and arginase activity. *gln3* mutations were epistatic to *ure2* mutations, suggesting that Ure2 acts as an antagonist of Gln3. Northern blots of *GLN3* mRNA or β-galactosidase assays of *URE2-lacZ* or *GLN3-lacZ* fusions, from strains grown in different nitrogen sources, revealed that gene expression of *URE2* and *GLN3* was not regulated at the level of transcription or translation by nitrogen source (Coschigano and Magasanik, 1991; Minehart and Magasanik, 1991). This evidence is consistent with the idea that Ure2 acts catalytically on Gln3 to regulate transcription of NCR-sensitive genes. Changes to *GLN3* copy number, and subsequently levels of Gln3 production, drastically affected expression of *GDH2*. Similar changes to Ure2 levels only had a small effect however, suggesting that Gln3 and Ure2 do not interact stoichiometrically (Coschigano and Magasanik, 1991; Blinder et al., 1996). Ure2 does not cause Gln3 to be degraded, as intracellular Gln3 levels were not decreased when *URE2* was overexpressed. Immunoprecipitated complexes of Gln3 and 5'-GATAAG-3'-containing DNA from cell extracts showed the same amount of Gln3 bound to DNA when Gln3 was overexpressed, independent of Ure2 expression levels. Therefore, Ure2 does not prevent binding of Gln3 to GATA sites, although Ure2 may decrease Gln3 binding to DNA or may directly or indirectly block the ability of Gln3 to activate transcription. Direct interaction between Ure2 and Gln3 would result in the formation of a complex containing both proteins, and coprecipitation of Ure2 and Gln3 was indeed recognised using anti-Gln3 serum (Blinder

et al., 1996). A non-stoichiometric interaction has previously been demonstrated (Coschigano and Magasanik, 1991; Blinder et al., 1996), however, and an alteration to Gln3 catalysed by Ure2 would still involve complex formation between Ure2 and Gln3. The similarity of Ure2 to glutathione *S*-transferases may provide clues to the mechanism of Ure2-mediated negative regulation of Gln3, perhaps by modification of Gln3 by glutathione attachment.

Courchesne and Magasanik (1988) reported increased levels of enzymes subject to regulation by Ure2 and Gln3, in response to increased glutamate availability, while use of proline as a nitrogen source only resulted in slightly increased enzyme levels. Gln1 activity and *GLN1-lacZ* expression were high when glutamate was the nitrogen source and low when glutamine was used, but always high in a *ure2* strain (Coschigano and Magasanik, 1991). Likewise, *GAP1* mRNA and Gap1-β-galactosidase production was low in the presence of glutamine, but high when glutamate, proline or urea was the nitrogen source. *gln3* mutants showed a 15- to 20-fold reduction in *GAP1* mRNA and Gap1-β-galactosidase production when grown on glutamate, and a two-fold decrease on urea (Stanbrough and Magasanik, 1995). NCR-sensitive genes are therefore activated by the Gln3/Ure2 cascade in the presence of glutamate and repressed when glutamine is present, while an independent activator is responsible for half of activation on urea, and all in the *gln3* mutant.

1.3.1.3 Dal80

Chisholm and Cooper (1982) identified a third regulator involved in NCR, Dal80. Mutations in *DAL80* resulted in over-production of inducer-independent allantoin pathway enzymes and transport proteins, indicating that Dal80 functions as a negative regulator. The amino acid sequence of Dal80 contained a GATA zinc-finger motif similar to Gln3, and a leucine zipper motif (Coornaert et al., 1992). Dal80 bound to a subset of UAS_{NTR} elements, URS_{GATA}, which consisted of a pair of GATA-containing sequences, oriented tail-to-tail or head-to-tail, 15 to 35 bp apart (Cunningham and Cooper, 1993). There is significant overlap between Gln3 and Dal80 binding sites, and Cunningham et al. (1994) have shown that both proteins bind to a GATA sequence upstream of *UGA4*, a site required for both Gln3-activation and Dal80-repression of *UGA4* expression. A possible mechanism for the opposing positive and negative

regulation by Gln3 and Dal5 could be competition for common regulatory elements. Daugherty et al. (1993) have carried out a broad survey of responses of NCR-responsive genes containing multiple UAS_{NTR}/URS_{GATA} elements, to *dal80* and *gln3* null mutations. Expression of all genes was increased in a *gln3* mutant. In a *dal80* mutant, expression ranged from a marked increase of *UGA1*, *CAN1*, *GAP1* and *DAL4*, less increase for *GDH1*, *PUT1*, *PUT2* and *PUT4*, to no change for *GDH2* and *GLN1*. Thus, more than just the presence of multiple UAS_{NTR} sequences may be involved in NCR and DAL80 responsiveness.

DAL80 itself has multiple UAS_{NTR}/URS_{GATA} elements in its upstream flanking region, and electrophoretic mobility shift assays (EMSAs) have demonstrated that Gln3 and Dal80 bind to these sequences (Coffman et al., 1997). Moreover, expression of *DAL80* is NCR-sensitive, and is regulated by Dal80, Ure2, Gln3, and two additional regulators, Gat1 and Deh1 (Cunningham and Cooper, 1991; Coffman et al., 1997). In *gat1* or *gln3* mutants, *DAL80* expression was reduced to barely detectable levels, and in double mutants, expression was virtually nonexistent (Coffman et al., 1997).

1.3.1.4 Gat1

If regulation of NCR-sensitive gene expression occurs exclusively by the Ure2/Gln3 cascade, then *ure2* or *gln3* mutations should abolish NCR-response. While *gln3* mutants showed a 15 to 20-fold reduction in mRNA and Gap1-β-galactosidase production when grown on glutamate, only a two-fold decrease was observed on urea (Stanbrough and Magasanik, 1995). Coffman et al. (1995) assayed *GAP1*, *CAN1*, *DAL5*, *PUT1*, *UGA4* and *GLN1* expression in single, double or triple mutants of *GLN3*, *URE2* and *DAL80*, grown with different nitrogen sources. *GAP1* mRNA levels in *gln3* or *gln3 dal80* mutants were two to four-fold less than wildtype when grown with proline, and transcripts were undetectable when the double mutant was grown with asparagine. In the triple mutant, *GAP1* mRNA was produced when proline, but not asparagine or glutamine, was used as a nitrogen source. A second pathway involved in regulation of NCR-sensitive genes therefore exists, and Ure2 is not the sole negative regulator, if at all, of this system. Transcript levels of four of the six genes showed similar responses to nitrogen source in the single, double and triple mutants, although the magnitude of the response differed, particularly in the triple mutant. Therefore, the

unidentified transcription factor does not participate in expression of all NCR genes to the same extent.

Coffman et al. (1996) reasoned that as UAS_{NTR} elements were required and sufficient for mediating NCR-sensitive expression, a second transcriptional activator would possess a GATA-binding zinc-finger motif. The ORF, *GAT1*, was predicted to encode a protein with such a motif, and has significant sequence similarity to Gln3. The transcriptional activation of a range of NCR-sensitive genes was compared in *gln3* and *gat1* single and double mutants, grown in a range of nitrogen sources. *GAP1* mRNA levels were reduced to half of wildtype levels in *gln3* or *gat1* mutants, and *DAL5* and *UGA4* transcript levels were reduced even more when proline was the nitrogen source. Transcript levels for *gln3 gat1* strains grown on this nitrogen source were undetectable, so Gln3 and Gat1 are both required for transcriptional activation in the presence of proline, but the extent to which each were required for different NCR-sensitive genes varies. When fused upstream of a reporter protein, Gat1 can weakly activate transcription. Stanbrough and Magasanik (1996) determined the ability of upstream regions of *GAP1* containing various deletions to serve as elements supporting activation of transcription by Gln3 or Gat1. They demonstrated that full response of both activators required the presence of two 5'-GATAAG-3' sites, and auxiliary sites located in an interval between 602 and 453 bp upstream of the translational start site. Gln3 and Gat1 can therefore utilise the same sites to activate expression.

The expression of *GAT1* itself is sensitive to NCR. Disruption of *DAL80* in a *ure2* background resulted in increased transcription of *GAT1*, and transcription was decreased, but still NCR-sensitive, in a *gln3 ure2* background. Putative Gln3 and Dal80 binding sites are present upstream of *GAT1*, and EMSAs showed an altered mobility of species after incubation of either protein with an oligonucleotide consisting of 70 bp of *GAT1* upstream sequence. Therefore, Gln3 and Dal80 do indeed bind to these regions. Furthermore, mutations in UAS_{NTR} or URS_{GATA} sites reduced binding (Coffman et al., 1996). Gat1 is therefore produced, and able to activate transcription of NCR-sensitive genes, in the presence of poor nitrogen sources such as proline and urea, whereas Gln3 best activates transcription when glutamate is the nitrogen source.

1.3.1.5 Deh1

A fourth transcription factor has been identified, Deh1, Nil2 or Gzf3, through similarity to Dal80 (Stanbrough et al., 1995; Coffman et al., 1997; Soussi-Boudekou et al., 1997). Like Dal80, Deh1 contains GATA zinc-finger and leucine zipper motifs, and negatively regulates some, but not all, NCR-sensitive genes. Deletion of *DEH1* had no effect on NCR-sensitive gene expression in proline. However, when glutamine was present, expression was increased for *GAT1* (Rowen et al., 1997), *GAP1*, *DAL80* (Soussi-Boudekou et al., 1997), and *UGA4*, but not for *PUT4* (Coffman et al., 1997). Like the previously described NCR transcription factors, EMSAs have demonstrated that Deh1 forms DNA-protein complexes with GATAA-containing *UGA4* and *GAP1* promoter fragments (Coffman et al., 1997). Experiments using *lacZ* fused to a synthetic UAS with three GATA sites have shown that Deh1 exerts its effects by competing with mostly Gat1, and partly Gln3, for UAS_{NTR} sites (Rowen et al., 1997). This is consistent with observations that deletion of *DEH1* partially relieved NCR-sensitivity of *GAP1-lacZ* expression, and deletion of *GAT1* abolished *GAP1-lacZ* expression, in yeast grown in the presence of glutamine (Soussi-Boudekou et al., 1997).

Like *GAT1* and *DAL80* expression, *DEH1* expression is also sensitive to the nitrogen source, and multiple GATA sequences are present upstream of *DEH1*. *DEH1* expression is largely Gln3-independent, partly Gat1-dependent, and most highly regulated by Dal80 (Coffman et al., 1997). In the presence of proline, Dal80 negatively regulates itself and *DEH1*, while in the presence of glutamine, Deh1 negatively regulates *DAL80* and *DEH1* expression (Coffman et al., 1997; Soussi-Boudekou et al., 1997).

Transcriptional control of NCR-sensitive gene expression is therefore regulated by an intricate, cross-regulatory system involving at least two positive transcriptional regulators, Gln3, Gat1, and two negative transcription factors, Dal80 and Deh1, all belonging to the GATA DNA-binding family (Figure 1.3). Gln3 is negatively regulated post-transcriptionally by Ure2 in response to high glutamine levels, and in *gln3 ure2* mutants, expression of NCR-sensitive genes remains NCR sensitive, providing evidence for the involvement of another protein acting, analogously to Ure2, on Gat1. Expression of *GAT1*, *DAL80* and *DEH1* is regulated by nitrogen source, although each gene is regulated at different levels by the different transcription factors. Dal80 and

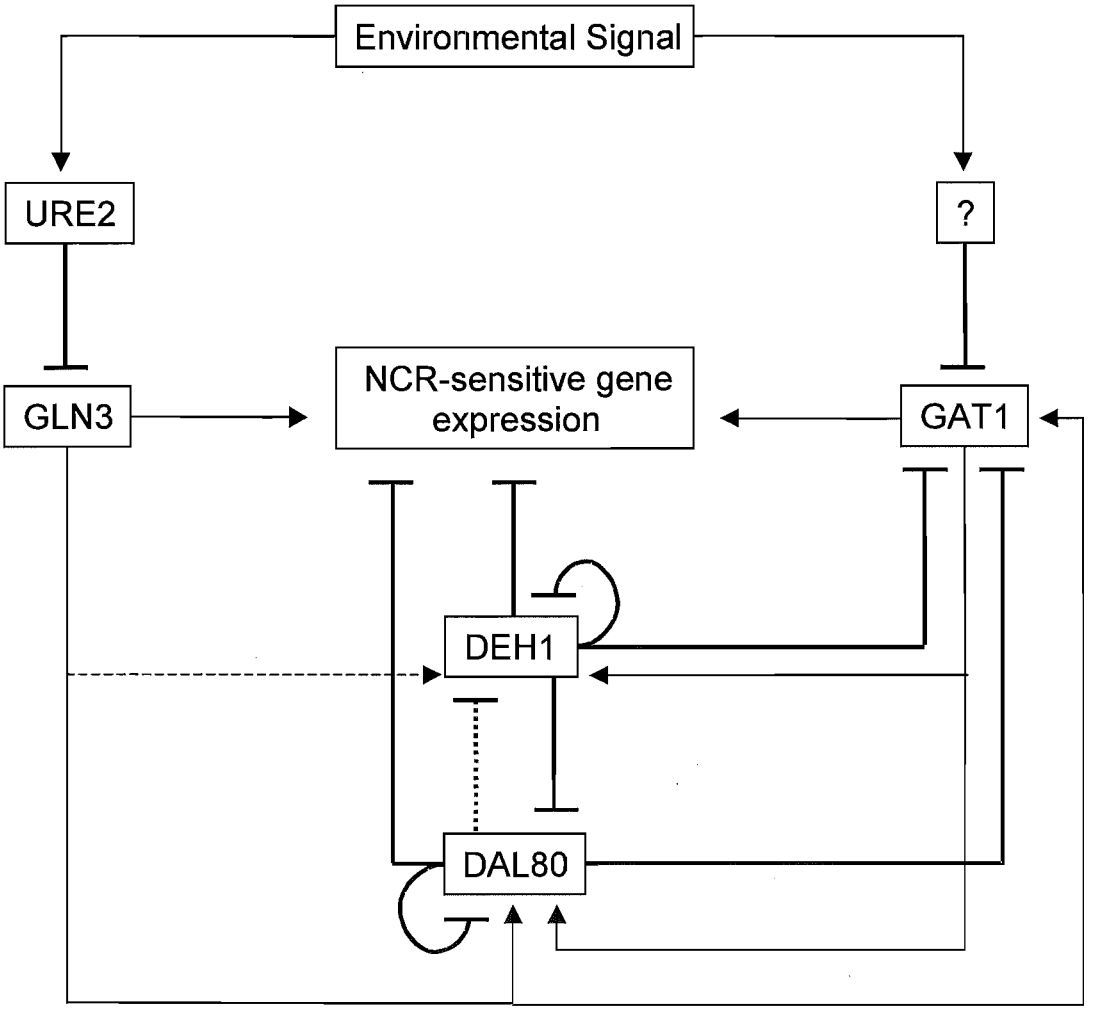

Figure 1.3: Model of the regulatory network involved in the expression and repression of Nitrogen Catabolite Repression (NCR)-sensitive genes in *S. cerevisiae*. Arrows represent positive regulation and bar-ended lines depict negative regulation. Dotted lines represent weak regulation. Adapted from Coffman et al., 1997.

Gat1 are produced, and thus regulate, in conditions of nitrogen limitation, whereas Deh2 is only slightly regulated by the Gln3/Ure2 cascade, so is produced, and hence functions, in conditions of nitrogen excess. The outcome of activation or repression of NCR-sensitive genes is therefore determined by competition between the positively and negatively acting transcription factors for binding to UAS_{NTR} sites, with transcription factor levels ultimately dictated by the quality of the nitrogen source. The various levels of control in this system therefore allow yeast to be highly responsive to nitrogen source quality and availability.

1.3.1.6 What is the Nitrogen Source Effector for NCR?

Although considerable information exists pertaining to the pathways involved in regulation of NCR gene expression, the role of the key metabolites as stimulators or inhibitors of these pathways still remains largely unknown. *GAP1* (Jauniaux and Grenson, 1990) and *PUT4* (Jauniaux et al., 1987) repression was partially lifted in *gln1*^{ts} and *gdhA* (partially glutamine auxotrophic) strains, grown in the presence of ammonium. These results imply that conversion of ammonia to glutamine is required to exert ammonium repression (Wiame et al., 1985). Mechanisms by which glutamine may effect regulation are entirely speculative. Wickner (1994) has demonstrated the ability of Ure2 to change into a prion-type conformation, Ure3. It is possible that glutamine may interact allosterically with a negative regulator such as Ure2, and perhaps alter Ure2 conformation. Experiments by ter Schure et al. (1995a, 1995b, 1998), apparently contradicting those of Jauniaux and Grenson (1990) and Jauniaux et al. (1987), suggested that NCR-sensitive gene expression was dependent on the concentration of ammonium, irrespective of glutamine concentration. In nitrogen-limited continuous cultures, increases in *GAP1*, *PUT4*, *GLN1* and *GDH1* expression correlated with decreasing extracellular ammonium concentration, even in a *gln1* mutant. The ammonium signal was specific for NCR-mediated gene expression and not a general stress response, as deletion of *URE2* in a *gln1-37* background prevented repression by ammonium. Ammonium did not induce redistribution of glutamate or glutamine between the cytosol and vacuole, either. A potential candidate for an ammonium sensor is the NCR-repressible Mep2 high affinity ammonium permease (Lorenz and Heitman, 1998a), and possibly also the Mep1 lower affinity ammonium permease (Lorenz and Heitman, 1998b). Lorenz and Heitman (1998a) suggested that the Mep2 system might serve as an ammonium sensor to regulate pseudohyphal growth.

Defects of a *mep2* variant were suppressed by increases in *GPA2*, *RAS2* or exogenous cAMP. Gpa2 and Ras2 regulate adenylate cyclase activity, which leads to activation of the cAMP-dependent protein kinase. The Mep2 permease may function in a signalling pathway upstream of Gpa2, Ras2 and cAMP, ultimately controlling phosphorylation by the kinase in response to changes in ammonium concentration. Diverse yeast metabolic processes are regulated by the Ras/PKA signal pathway and Sáenz et al. (1997) have reported that this pathway is involved in regulation of L-leucine transport through Bap2 and S2 permeases, in response to the nitrogen source present. As transport through plasma membrane permeases is the first step in the metabolism of any nutrient, the permease is in a unique position to both sense and import substrates. Evidence exists of other permeases in yeast also acting as sensors of nutrient availability, for example, glucose permease sensors (Özcan et al., 1996).

1.3.2 Nitrogen Catabolite Inactivation (NCI)

A second, post-translational, mechanism called Nitrogen Catabolite Inactivation (NCI) is involved in the negative regulation of several yeast amino acid transporters, for example the Gap1, Dal5, and Put4 permeases (Grenson, 1983a), in response to nitrogen source. Grenson (1983a) has shown that when ammonium was added to wildtype, proline-grown yeast, the Gap1 permease was rapidly and completely inactivated, so as to be completely lost within one hour. Upon addition of ammonium to the medium of strains containing at least one of the mutations *npi1* (*mut2*), *npi2* (*mut4*), or *pgr*, pre-existing Gap1 activity remained constant, and further increases in Gap1 activity were prevented. These results suggested that while Gap1 was no longer synthesised, inactivation of already synthesised Gap1 was not occurring. The method of regulation that is no longer present in *npi1*, *npi2* and *pgr* mutants therefore appears to be distinct from NCR. Further evidence, discussed subsequently, also supports this prediction. While inactivation still occurred upon addition of ammonium to *ure2* and *gln1*^{ts} mutants that alleviate NCR-sensitive gene expression, in double mutants of *ure2* or *gln1*^{ts} and *npi1, npi2* or *pgr*, Gap1 activity was as high as in proline-grown yeast. Also, whilst *npi1* and *npi2* mutations relieve permease inactivation, Jauniaux et al., (1987) have shown that they do not affect expression of NCR-regulated *PUT4*. Furthermore, a high level of *GAP1* transcript only resulted in a correspondingly high level of permease

activity when urea was used as a nitrogen source (Stanbrough and Magasanik, 1995). In glutamate-grown *GLN3* cells, and glutamate or glutamine-grown *ure2* cells, very high mRNA levels did not correlate with low permease activity. Glutamate-grown, wildtype yeast had 70-fold less permease activity than urea-grown cultures of the same strain.

Results by Stanbrough and Magasanik (1995) provide convincing evidence that the nitrogen source-dependent, non-NCR control of Gap1 occurs post-transcriptionally. They have also demonstrated that this control is not mediated at the translational level. Glutamate and urea-grown yeast produced similar amounts of Gap1 fused to a 9-amino acid epitope of influenza hemagglutinin protein HA1. Also, the total amount of Gap1 present did not correlate with total permease activity, so some was present in an inactive form. Gap1 antibodies bound to bands bigger than Gap1 on a Western blot, thus Gap1 may be covalently modified. Hein et al. (1995) have revealed that ammonium inactivation of the Gap1 permease is accompanied by its degradation, and a functional *NPI1* gene product is required for both inactivation and degradation of Gap1. Upon addition of ammonium to proline-grown yeast, citrulline uptake by Gap1 decreased rapidly and completely, whereas preexisting Gap1 activity was preserved in *npi1* mutants. Western blots from cell extracts showed that Gap1 levels were still high in *npi1* mutants two hours after addition of ammonium to media, but almost nonexistent in the wildtype. There was an apparent delay between loss of Gap1 uptake activity and disappearance of immunoreactive Gap1 protein, thus Gap1 could be inactivated and subsequently degraded. Hein et al. (1995) cloned the *NPI1* gene and showed that it encoded the essential ubiquitin ligase, Rsp5, a member of the E6-AP-like family of ubiquitin ligases. The human ubiquitin ligase E6-AP interacts with the E6 protein of the human papillomavirus, and once bound, the E6/E6-AP complex binds to and targets the tumour suppressor protein p53 for ubiquitin-mediated proteolysis. Rsp5 may therefore regulate inactivation of amino acid permeases in response to ammonium in growth media, in a similar, ubiquitin-dependent manner.

1.3.2.1 Ubiquitination

The ubiquitin pathway involves the covalent attachment of the 76 amino acid ubiquitin polypeptide to target proteins through isopeptide bond formation between the carboxyl terminus of ubiquitin and the ε–amino group of one or more lysine residues on the

substrate. Subsequently, additional ubiquitin molecules can be attached via lysine residues of ubiquitin itself, forming a multiubiquitinated substrate, which is recognised and degraded by the proteasome or vacuole. A cascade of steps is involved in the ubiquitin pathway, initially involving activation of ubiquitin by an E1, or ubiquitin-activating, enzyme. This activation reaction requires ATP hydrolysis, resulting in the formation of a high-energy thioester bond between a specific cysteine residue of the enzyme and the carboxyl terminus of ubiquitin. At least two E1 proteins (consisting of the products of the *UBA1* and *UBA2* genes) have been recognised in yeast. Ubiquitin is then transferred to a cysteine residue of an E2, or ubiquitin-conjugating, enzyme, at least 13 of which have been recognised in yeast. E2 is thought to catalyse ubiquitination of the substrate, often with the assistance of a third enzyme, E3, or ubiquitin ligase. E3 is thought to be required for substrate recognition, perhaps by substrate binding, and has been proposed to form an ubiquitin-thioester intermediate, thus may catalyse isopeptide bond formation between ubiquitin and the substrate. Including Rsp5, at least five E3 proteins have been identified in yeast (Hershko and Ciechanover, 1992; Ciechanover, 1994; Hicke, 1997; Yamao, 1999).

1.3.2.2 Rsp5

Rsp5 appears to have a general role in permease turnover, rather than specific to regulation of a permease according to nitrogen source. Hein et al. (1995) showed that Rsp5 is required for degradation of the uracil permease, Fur4. Stress-induced degradation of Fur4 caused a sharp drop in uracil transport and immunodetected Fur4 permease levels in the wildtype, whereas no loss to uracil uptake and no permease degradation occurred in the *npi1/rsp5* mutant. The uracil permease was tagged by ubiquitin *in vivo*, and ubiquitin-permease conjugates were present in wildtype cells, but barely detectable in *npi1/rsp5* mutants. Moreover, there was a direct correlation between permease ubiquitination and removal from the membrane (Galan et al., 1996). Endocytosis is dependent on *END3* and *END4* gene products, also required for endocytosis of pheromone receptors (Raths et al., 1993). Degradation follows endocytosis, and uracil permease degradation occurs in the vacuole and not the proteasome. Permease turnover was greatly reduced in *pep4* mutants which have a defective vacuolar protease, and the permease was not stabilised in *pre1 pre2*, *cim3* and *cim5* mutants that have impaired catalytic (*pre*) or regulatory (*cim*) proteasome subunits (Galan et al., 1996). The uracil permease contains a 9-residue sequence similar to the

destruction box required for conjugation of ubiquitin to cyclins, and a permease having an arginine to alanine mutation within this sequence was resistant to stress-triggered degradation (Galan et al., 1994). Internalization by endocytosis and subsequent vacuolar degradation of the maltose permease, triggered by impaired protein synthesis and the presence of a fermentable substrate, also requires Rsp5 (Lucero and Lagunas 1997). Rsp5 therefore appears to be involved in ubiquitination-mediated internalization and degradation of a number of yeast membrane permeases, and a similar mechanism was proposed for the ammonium-triggered down-regulation of amino acid permeases (Figure 1.4A). Evidence provided by Springael and André (1998) supports this proposal. Cell membrane immunoblots detecting Gap1, and assays of Gap1 activity, showed that an *act1-1* mutant, defective in endocytosis, had strongly impaired ammonium-induced inactivation of Gap1. An internalization step therefore appears to be required for ammonium-triggered inactivation of Gap1. In *pep4* mutants, Gap1 was strongly protected against ammonium-triggered degradation, thus, like the uracil and maltose permeases, degradation of Gap1 proceeding endocytosis occurs in the vacuole. Gap1 is ubiquitinated upon addition of ammonium to proline-grown yeast cultures, and this ubiquitination requires Rsp5. Immunoblots detecting Gap1 when cells were grown in the presence of proline contained a major band migrating the same distance as Gap1, and faint bands corresponding to the size of one or two ubiquitin molecules attached to Gap1. When ammonium was added to cells, the major Gap1 band decreased, and intensities of predicted mono-, di-, and tri-ubiquitinated Gap1 bands increased considerably. In *npi1/rsp5* strains, no ubiquitinated Gap1 bands were observed, thus both basal and ammonium-stimulated ubiquitination requires Rsp5. Strains containing a mutation within ubiquitin of the lysine 63 residue, responsible for forming multi-ubiquitin chains on Gap1, still contained Gap1 molecules attached to either one or two ubiquitin moieties, thus Gap1 appears to have two sites for ubiquitination attachment.

Many features of Rsp5, essential for its role in ubiquitination, have been identified. Rsp5 has a putative Ca^{2+}-dependent phospholipid interaction motif (C2) in its N terminus which may be involved in targeting membrane bound proteins, and can interact with proteins and inositol polyphosphates. The C2 motif is followed by three to four repeats of a WW domain, a protein motif that has an affinity for proline-rich sequences with the consensus binding site containing a PPxY sequence (Einbond and Sudol, 1996), and is implicated in mediating protein-protein interactions. In the C-

terminus, Rsp5 contains a potentially catalytic HECT (Homologous to the E6-AP Carboxyl Terminus) domain. Huibregtse et al. (1995) have demonstrated that Rsp5 is able to form thioesters with ubiquitin *in vitro*, and mutation of the cysteine residue in the HECT domain, conserved within E6-AP-related proteins, eliminates this ubiquitin-binding ability. Changing a conserved leucine to serine within the HECT domain, truncation of the carboxyl-terminal six amino acid residues of Rsp5, and a mutation of the conserved cysteine to alanine, all impaired ubiquitin-thioester formation and catalysis of substrate ubiquitination. Rsp5 bound to one of its substrates, Rpb1, despite these mutations. The approximately 350 amino acid long HECT domain was sufficient for ubiquitin-thioester formation (Wang et al., 1999). Moreover, Springael et al. (1999a) have investigated the roles of the different domains of Rsp5 in ammonium-triggered down-regulation of Gap1. The conserved cysteine was required for cell viability, as well as ubiquitination and subsequent endocytosis of Gap1. Rsp5 containing a replacement of the conserved cysteine by serine, could not complement the inability of a *npi1/rsp5* mutant to ubiquitinate and down-regulate Gap1 in the presence of ammonium. Addition of a plasmid encoding Rsp5, lacking the N-terminal C2 domain, had a different effect. Gap1 remained active and strongly protected from ammonium-triggered degradation, but was ubiquitinated to some extent. The C2 domain may therefore be responsible for endocytosis of ubiquitinated permeases, perhaps by mediating targeting of Rsp5 to regions of the plasma membrane that actively endocytose, or to some other specific membrane compartment. Wang et al. (1999) have confirmed that the WW domains play a role in recognition of at least some substrates of Rsp5, and mutations of the WW domains three and two partially and completely abolished, respectively, binding of Rsp5 to Rpb1. Proteins containing a complete deletion of the HECT domain could bind Rpb1 as well as wildtype Rsp5 proteins.

1.3.2.3 Gap1 Requirements for NCI

The regions of Gap1 essential for its ammonium-triggered, ubiquitin-dependent degradation have been studied. Mutations in Gap1, including substitution of a C-terminal di-leucine motif by two alanines, deletion of the last eleven amino acids of Gap1, or a single glutamate to lysine substitution within the sequence EEKAI (the *pgr* mutation described by Grenson, 1983a), abrogated the ammonium-triggered down-regulation of Gap1 (Hein and André, 1997). These mutant proteins were all ubiquitinated following ammonium addition, thus protection from ammonium-triggered

degradation may arise due to less efficient ubiquitination. Alternatively, down-regulation may be affected at a step subsequent to ubiquitination (Springael and André, 1998). These mutations all occurred in a region predicted to form an α-helix, on the cytoplasmic side of the membrane. The last eleven amino acids of Gap1 comprise a region rich with aromatic amino acids, and are conserved between other yeast amino acid permeases (Hein and André, 1997). Di-leucine motifs have been found to act as a signal for internalization of several eukaryotic membrane proteins (Haft et al., 1994; Dittrich et al., 1996), and are involved in directing membrane proteins to the endosome and lysosome (Sandoval and Bakke, 1994). The EEKAI sequence is reminiscent of the DAKSS sequence present in the cytosolic tail of the Ste2 receptor, the lysine residue of which is essential for ubiquitination and subsequent endocytosis of Ste2 (Hicke and Riezman, 1996). EAKSS and DAKAS mutations allow efficient endocytosis, but AAKSS does not (Rohrer et al., 1993). Although the C-terminal region of Gap1 is required for ammonium-induced inactivation, replacement of the C-termini of Fur4 and Can1 permeases by the last 47 amino acids of Gap1 is not sufficient to make these permeases sensitive to ammonium-induced down-regulation (Hein and André, 1997).

1.3.2.4 Npi2

The operation of the ubiquitin pathway also requires recycling of ubiquitin, which is carried out by ubiquitin C-terminal hydrolases (E4 enzymes), 16 members of which have been identified in *S. cerevisiae*. E4 enzymes cleave ubiquitin from the protein substrate before and during proteolysis. *NPI2* has been shown to be allelic to *DOA4*, which encodes a member of the E4 family (Springael et al., 1999b). Mutations in *NPI2/DOA4* have pleiotropic effects, some of which include an approximately four-fold decrease in monomeric ubiquitin levels (Springael et al., 1999b), and strong inhibition of ubiquitination, internalization and degradation of the maltose (Lucero and Lagunas, 1997; Medintz et al., 1998), uracil (Galan and Haguenauer-Tsapis, 1997) and Gap1 permeases (Springael et al., 1999b). These mutant phenotypes could be overcome by overexpression of an ubiquitin-coding gene. Therefore, Npi2/Doa4 plays a more general role in ubiquitin-mediated protein turnover, rather than a specific regulatory role of negatively regulating amino acid permeases in response to nitrogen source.

No E1 or E2 candidates have been identified in the nitrogen source-triggered, ubiquitin-mediated degradation of yeast amino acid permeases. Rsp5 has been shown *in vitro* to bind to members of the E2 UBC4/UBC5 subfamily, including the human protein UbcH5 (Nuber et al., 1996; Kumar et al., 1997). *In vitro*, Nuber and Scheffner (1999) have used chimeric E2s generated between UbcH5 and other similar E2 proteins, to demonstrate that a region encompassing the catalytic site cysteine residue is essential for the ability to interact with Rsp5. Also required was a phenylalanine residue, conserved throughout members of the E2 UBC4/UBC5 subfamily, but not present in other described E2 proteins. The *S. cerevisiae* proteins, Ubc4 and Ubc5, are very similar to the respective proteins in humans and are involved in stress-related functions (Seufert and Jentsch, 1990), thus may be candidates for the E2 protein involved in Rsp5 and ubiquitin pathway-dependent down-regulation of Gap1 in the presence of ammonium. Moreover, de la Fuente et al. (1997) have demonstrated that Ubc4 is required in the Rsp5 and ubiquitin-dependent degradation of an inhibitory protein of the plasma membrane proton-ATPase in response to glucose.

1.3.2.5 Nitrogen Permease Reactivation (NPR)

The sole positive regulator identified in the post-translational NCI system for permease regulation comprises the nitrogen permease reactivator protein, Npr1. Mutations affecting the *NPR1* locus have a pleiotropic depressing effect on the activity of at least five ammonium-sensitive uptake systems, Gap1, Put4, Dal5, Mep1 and Mep2. Permease inactivation was 50-fold to complete, depending on the system, and independent of the nitrogen source present (Grenson and Dubois, 1982). In the presence of proline, *npi1/rsp5*, *npi2*, or *pgr* mutations restore Gap1 activity to *npr1* mutants. Gap1 activity was not reinstated in *npr1* strains containing one of the following mutations relieving NCR-sensitive gene expression, *mep1*, *gdh1*, *gln1*^{ts} or *ure2*. Grenson (1983b) therefore predicted that the product of *NPR1* was necessary for Gap1 reactivation or protection from inactivation by the products of *NPI1/RSP5* and *NPI2*, in the presence of proline. Consistent with a non-NCR-mediated role for nitrogen regulation by Npr1, northern analyses revealed that *GAP1* mRNA synthesis was not inhibited in a *npr1* strain, in either repressing or nonrepressing nitrogen sources. Indeed, *npr1* mutants had slightly promoted *GAP1* expression when grown in the presence of ammonium compared with the wildtype (Vandenbol et al., 1990). Similarly, *PUT4* mRNA levels were increased in *npr1* mutants grown in ammonium

medium (Jauniaux et al., 1987), as was the activity of several nitrogen catabolic enzymes (Grenson, 1983b). The effect of *NPR1* mutation on gene regulation was probably indirect. Npr1 positively regulates at least two ammonium permeases, and ammonium uptake is repressed in *npr1* mutants (Grenson and Dubois, 1982), thus expression of genes sensitive to ammonium levels would be increased in these mutants. Permease regulation by Npi2 and Rsp5 is not restricted to nitrogen-regulated permeases, thus Npr1 may be the candidate responsible for regulating permease inactivation/reactivation in response to nitrogen source.

Vandenbol et al. (1987) have cloned and sequenced *NPR1*. Npr1 contains consensus sequences characteristic of serine/threonine protein kinases, and has strong amino acid similarity to Wee1, a protein kinase involved in mitotic control in *Schizosaccharomyces pombe* (Vandenbol et al., 1990). The predicted carboxy-terminal region of Npr1 contains Gly-X-Gly-X-X-Gly residues thought to be involved in ATP-binding, and a conserved lysine which is thought to be involved directly in the phosphotransfer reaction. Downstream from these regions, three other protein kinase consensus sequences are present. Similarities of Npr1 to serine-threonine kinases suggest that regulation by Npr1 may be mediated by phosphorylation. Experiments carried out by Stanbrough and Magasanik (1995) revealed a strong correlation between dephosphorylation and inactivation of Gap1. Nine-fold greater levels of phosphorylated Gap1 were isolated from urea-grown than glutamate-grown cells, and Gap1 activity was 15-fold higher when cells were grown in urea. Addition of glutamine to growth media caused a rapid loss of Gap1 activity. Within five minutes of glutamine addition, phosphorylation of Gap1 was lost. A scheme proposed to explain Npr1-mediated regulation involves permease phosphorylation by Npr1 in the presence of poorly utilised nitrogen sources, thereby protecting the permease from degradation (Figure 1.4B). Upon a shift to readily utilisable nitrogen sources, Npr1 is inactivated, and no longer able to maintain permeases in a phosphorylated state, resulting in permease degradation. Due to the rapid dephosphorylation of Gap1 upon addition of repressing nitrogen sources, this model assumes that permease phosphorylation is short-lived in the absence of Npr1. That the phosphorylation state of a protein mediates its degradation by the ubiquitin pathway is not novel. Unlike the proposed, phosphorylation-mediated protection of Gap1 from degradation, serine and/or threonine phosphorylation of the

uracil permease, several cyclins, transcriptional regulators, and the a-factor receptor is a trigger for ubiquitination and down-regulation of these proteins (Hicke, 1997).

Mutations in *PER1* (Courchesne and Magasanik, 1983) and *AUA1* (Sophianopoulou and Diallinas, 1993) release Gap1 from inactivation in the presence of ammonium, but not glutamine and glutamate, and *GAP1* transcription is unaffected in *aua1* mutants. These results provide evidence that at least two post-transcriptional inactivation pathways exist. *AUA1* is itself weakly ammonium repressible (two- to three-fold), and derepressed in proline- or glutamate-containing media. Rousselet et al. (1995) have isolated a second positive regulator of amino acid transport acting at the post-translational level, Npr2. Mutations in *NPR2* affect urea and proline transport capacities, without affecting transcription of the genes encoding the proline and urea permeases, *PUT4* and *DUR3*. Transcription of *NPR2* is insensitive to NCR, and is transcriptionally activated in media containing proline or urea.

1.3.2.6 What is the Nitrogen Source Effector for NCI?

How does the inactivation/reactivation process respond to the quality of the nitrogen source? Early models predicted that Npr1 was degraded or not produced in the presence of good nitrogen sources. Transcriptional levels of *NPR1* are unaffected by nitrogen catabolite repressing conditions, so nitrogen regulation of Npr1 function must occur after transcription (Vandenbol et al., 1987). The amount of Npr1 detected on Western blots did not increase after a shift from readily utilisable nitrogen sources to nitrogen starvation conditions, suggesting Npr1 is not regulated at the level of protein stability, either (Schmidt et al., 1998). In poor nitrogen sources, or carbon or nitrogen starvation, the Tat2 permease is down regulated by ubiquitin pathway-mediated targeting to the vacuole. Gap1 and Tat2 are therefore inversely regulated. Schmidt et al. (1998) have provided evidence that implicates the TOR nutrient signalling pathway in this regulation of Tat2, via Npr1. In readily utilisable nitrogen sources and nonstarvation conditions, the TOR signalling pathway involving the Tor1 and Tor2 kinases (collectively called TOR), activates cell growth in response to nutrient availability, and inhibits starvation induced turnover of Tat2. TOR and Npr1 have antagonistic functions as Npr1 can suppress growth due to reduced tryptophan transport when TOR is inhibited, and loss of *NPR1* restored growth of yeast lacking TOR function. Tryptophan import was severely reduced, inhibiting cell growth in *tor2-1* mutants when *NPR1* was overexpressed, or

when Tor2 was inhibited by rapamycin. In *npr1* mutants, tryptophan import was increased, thus Npr1 negatively regulates the Tat2 permease when TOR function is reduced. As inhibitory effects on Tat2 transport are only observed when TOR function is reduced, Npr1 may be regulated by a TOR-dependent post-translational mechanism. HA-tagged Npr1 migrated slower when isolated from non-starved cells than from starved cells. When Npr1 from non-starved cells was treated with phosphatase, it migrated the same distance as Npr1 from starved cells. Dephosphorylation of Npr1 and downregulation of Tat2 rapidly followed inhibition of TOR by rapamycin in cells grown in non-starvation conditions. TOR therefore appears to control Npr1 function by Npr1 phosphorylation in non-starvation conditions. TOR controls translation initiation of many proteins in response to nutrients, via an additional protein, Tap42, thus Tap42 may help to control Npr1 phosphorylation. At nonpermissive temperatures, the temperature sensitive *tap42-11* mutant conferred a phenotype similar to TOR deficiency or inhibition, and Western blots showed a decrease in Tat2 levels. Tap42 also plays a role in maintaining Npr1 in a phosphorylated state in good nutrient conditions. Mutations in *TAP42* are epistatic to *TOR* mutations. Schmidt et al. (1998) therefore proposed the following model to explain regulation of Tat2 by Npr1, TOR and Tap42 (Figure 1.5). In the presence of nutrients, TOR activates Tap42 directly or indirectly, resulting in rapid phosphorylation and inactivation of Npr1. Npr1 cannot phosphorylate Tat2, thus Tat2 is not degraded. In absence of nutrients, TOR and Tap42 are inactive, so Npr1 is dephosphorylated and activated, and Tat2 is phosphorylated and degraded. Therefore, Gap1 and Tat2 are both phosphorylated by a Npr1-dependent mechanism under similar conditions. Whereas phosphorylation protects Gap1 from inactivation, Tat2 phosphorylation triggers Tat2 degradation. It remains to be determined whether phosphorylation of Npr1 as a result of the involvement of the TOR pathway and Tap42 in nutrient rich conditions, determines the ability of Npr1 to phosphorylate, and hence protect Gap1 from degradation. Npr1 also appears to be involved in regulation of the spermidine permease. Disruption of *NPR1* resulted in a three-fold decrease in spermidine transport when yeast was grown in ammonium or rich media, but almost no decrease in proline-containing media (Kaouass et al., 1998). These results suggest that Npr1 protects the spermidine permease from degradation in the presence of ammonium, thus phosphorylation of Npr1 in these conditions by the TOR pathway and Tap42 does not eliminate the ability of Npr1 to carry out all of its pleiotropic functions. Assuming

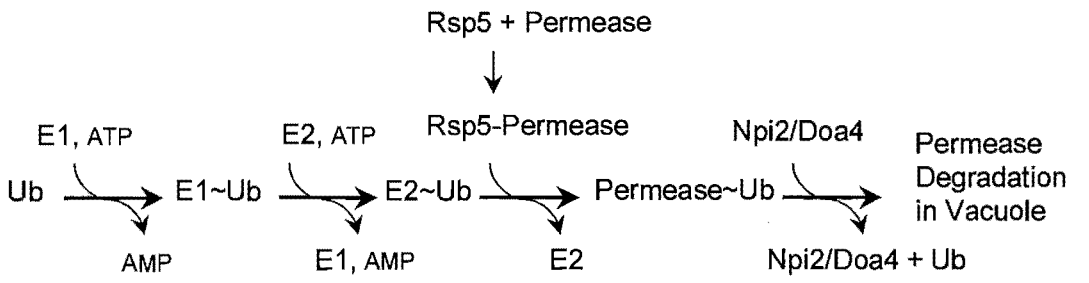

Figure 1.4A: Proposed ubiquitin-conjugation-dependent mechanism for regulation of permeases. In an ATP-dependent reaction, ubiquitin (Ub) is activated by an ubiquitin-activating enzyme (E1), forming a high-energy thiolester bond (E1~Ub). Activated ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2), forming an E2-ubiquitin thiolester (E2~Ub). Finally, ubiquitin is transferred to the permease substrate, in a reaction requiring substrate recognition by Rsp5. Once ubiquinated, the permease is targeted for degradation, and Npi2/Doa4 mediates recycling of Ub.

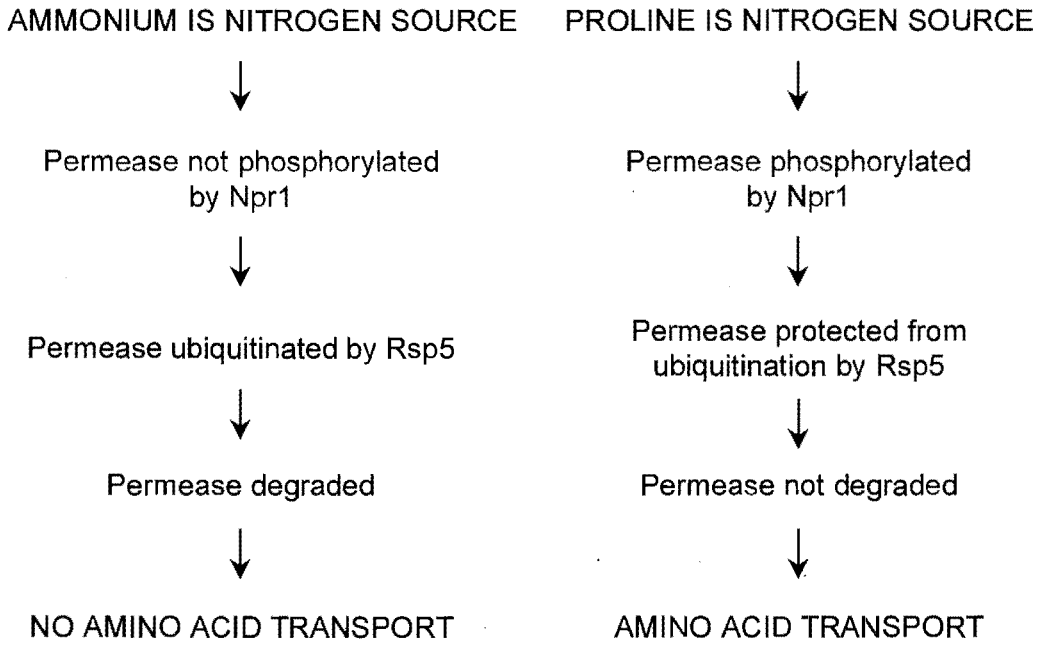

Figure 1.4B: Proposed outcomes to amino acid uptake mediated by permeases regulated by Nitrogen Catabolite Inhibition (NCI) and Nitrogen Permease Reactivation (NPR) in conditions where proline or ammonium is the nitrogen source. Permease function is ultimately determined by the winner in a competition between permease phosphorylation by Npr1 and permease ubiquitination by Rsp5.

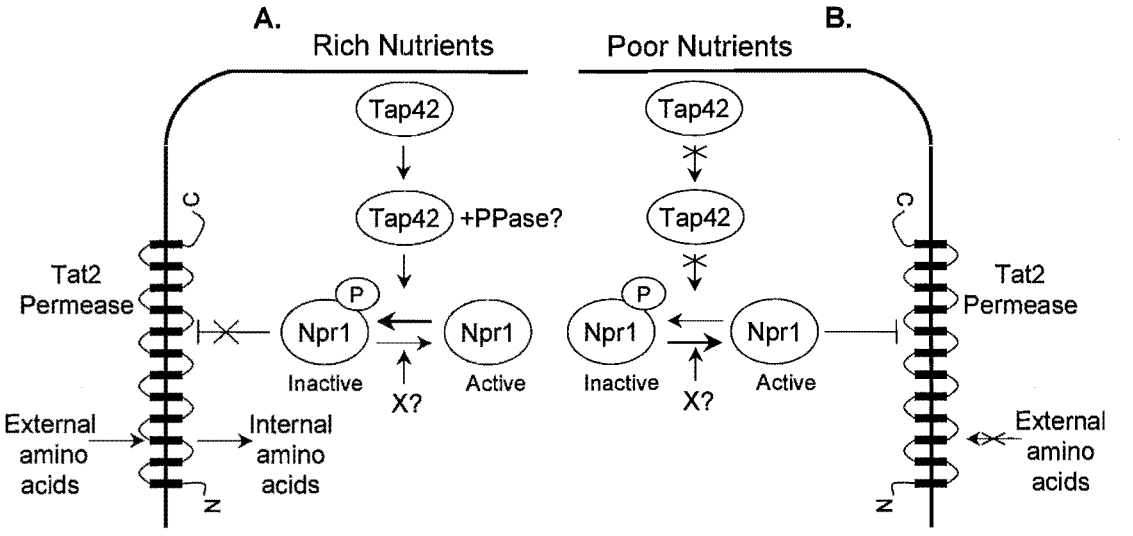

Figure 1.5: Model for regulation of Npr1, and subsequently Tat2, by TOR signalling pathway in response to external nutrients. In the presence of nutrients (A), the TOR pathway prevents Tat2 degradation by keeping Npr1 in an inactive, phosphorylated state. In starvation conditions (B), Npr1 is no longer regulated by the TOR pathway, thus is dephosphorylated by an unknown phosphatase, X, and negatively regulates Tat2. It is not known whether an additional phosphatase (PPase) is required for Npr1 phosphorylation. Arrows represent positive regulation and bar-ended lines depict negative regulation. Adapted from Schmidt et al., 1998.

that Npr1 directly phosphorylates Tat2 and Gap1, a second question is raised relating to how Npr1 phosphorylation protects Gap1 from ubiquitination, but triggers Tat2 degradation.

1.3.3 Amino Acid Induction

The availability of amino acids in the environment may also induce amino acid uptake by yeast. Verma et al. (1984) found that serine uptake by yeast was induced by addition of serine to media. More recently, Didion et al. (1996) have demonstrated that addition of branched-chain amino acids to minimal media enhanced transport of branched-chain amino acids. Expression levels of a *BAP2-lacZ* fusion in a strain grown in minimal ammonium (MA) medium and minimal proline (MP) medium were 5% and 1.6%, respectively, of that measured from yeast growing in synthetic complete (SC) medium. The availability of amino acids differentiates SC from minimal media, thus, *BAP2* transcription may be induced by the amino acids present. A survey of the effects of

various amino acids on Bap2-lacZ production revealed that all amino acids tested could enhance production of β–galactosidase activity to some extent. When added to MA medium, the branched chain amino acids, leucine, isoleucine and valine induced highest activity levels, at 75%, 60% and 70% of SC levels, respectively. Bap2 induction by amino acids is dependent on the nitrogen source present, with highest levels of induction by leucine occurring in the presence of ammonium, less in the presence of glutamine, and no induction was observed when proline was the nitrogen source. Similarly, de Boer et al. (1998), Iraqui et al. (1999a) and Barnes et al. (1998) have reported that expression of *BAP3*, *AGP1*, *TAT1*, *TAT2*, *GNP1* and a gene encoding a peptide transporter, *PTR2*, was also induced by addition of branched chain amino acids to growth media. Moreover, transcription of *BAP3* and *AGP1* was induced to various extents by most of the 20 naturally occurring amino acids, excluding proline, lysine, arginine, and histidine. Different media may have global effects on cell physiology, and it is possible that gene expression is universally increased when amino acids are available. A survey investigating amino acid inducibility of a wide range of amino acid permease genes showed that expression of many, for example *GAP1*, *MUP1* and *MUP3*, remained unchanged when amino acids were added to MA (Iraqui et al., 1999a). Thus, amino acid availability does not universally activate gene transcription.

1.3.3.1 Stp1

Mutations in *STP1*, also called *BAP1* and *SSY2*, resulted in reduced branched-chain amino acid uptake independent of the Gap1 permease (Tullin et al., 1991; Jørgensen et al., 1997; Jørgensen et al., 1998), leading earlier researchers (Tullin et al., 1991) to propose that this gene encoded a branched-chain amino acid permease (Tullin et al., 1991). The sequence of *STP1* suggests, however, that it is a previously characterised gene, the product of which is involved in the splicing of five families of pre-tRNAs. Localisation of Stp1 to the nucleus and the presence of three zinc-finger consensus sequences within the Stp1 sequence led researchers to investigate whether this protein functions as a transcriptional activator of branched-chain amino acid permease genes. Indeed, expression of a *BAP2-lacZ* fusion was low in a *stp1* mutant, and no longer amino acid-inducible, suggesting that Stp1 plays a positive role in induction of *BAP2* (Jørgensen et al., 1997). As tyrosine, tryptophan and phenylalanine transport was also decreased in *stp1* mutants, Stp1 may be involved in amino acid-dependent

transcriptional activation of additional permeases. Indeed, de Boer et al. (1998) have seen that amino acid induction of *BAP3* expression requires Stp1. The portion of the *BAP3* promoter necessary and sufficient for Stp1-dependent induction was isolated by assaying induction of reporter activity in the presence of amino acids, when the *BAP3* promoter, containing various deletions, was fused to *GUS* and *lacZ*. Sequences similar to this amino acid-dependent upstream activator sequence (UAS_{aa}), -418 to –392 bp relative to the *BAP3* 5'-ATG-3' start site, have also been found upstream of *BAP2*, *TAT1* and *PTR2*. Gel retardation assays have recovered no evidence of Stp1 binding to the BAP3 promoter region *in vitro*, however, thus Stp1 may indirectly regulate permease synthesis.

1.3.3.2 Ssy1: An Amino Acid Sensor?

A screen for mutants with reduced uptake of leucine, isoleucine and valine has also uncovered variants with mutations in *SSY1* (Jørgensen et al., 1998). *ssy1 gap1* mutants had a similar phenotype to *agp1 gap1* mutants, consistent with Ssy1 requirement for Agp1 function, and indeed, *AGP1* expression was no longer induced by amino acids in the *ssy1* mutant. *agp1 gap1* mutants still displayed residual, inducible, amino acid transport which was eliminated by deletion of *SSY1*, and a growth deficiency caused by the *ssy1* mutation was broader than in the *agp1* mutant. Ssy1 may therefore be regulating additional pathways to Agp1. A broad survey comparing RNA levels of different amino acid permease genes from wildtype and *ssy1* strains, grown in the presence of inducing levels of amino acids, indicated that Ssy1 is also required for the transcriptional induction of *BAP3*, *GNP1*, *BAP2*, *TAT1* and *TAT2*. Transcription of *MUP1*, *MUP3*, and several other genes encoding putative amino acid permeases with unknown specificity was unaffected by the *ssy1* mutation, however (Iraqui et al., 1999a).

Sequence information suggests that *SSY1* belongs to the major family of genes encoding amino acid permeases in yeast. Ssy1 is most similar to Lyp1, the high affinity lysine permease, although 241 amino acids larger than Lyp1, corresponding to a hydrophilic N-terminal extension and an approximately 30 amino acid insertion within extracellular regions connecting the seventh and eighth predicted transmembrane domains. These extensions have not been found in other amino acid permeases (Jørgensen et al., 1998). The codon bias index of Ssy1 is considerably lower than for other amino acid

permeases, suggesting that *SSY1* is expressed at much lower levels. Interestingly, these features distinguishing Ssy1 from other members of the AAP family are the same characteristics that differentiate the glucose permease sensors Snf3 and Rgt2 from other proteins of the sugar transport family (Özcan et al., 1996). *SSY1* may therefore encode an external sensor of amino acids, analogous to the glucose sensors and the proposed ammonium sensor, Mep2 (Lorenz and Heitman, 1998a). de Boer et al. (1998) have demonstrated that addition of citrulline to minimal media could induce expression of UAS_{aa}-*lacZ* and *BAP3* in *gap1* mutants, unable to transport citrulline. Similarly, *AGP1* is induced by extracellular rather than intracellular tryptophan levels, as *AGP1* was induced by tryptophan at equally strong levels in a wildtype strain and in a mutant largely deficient in tryptophan uptake (Iraqui et al., 1999a). Also, induction of *BAP2* by leucine still occurs in a strain largely deficient in leucine uptake (Didion et al., 1998). Furthermore, initial uptake of ^{14}C-labelled leucine, isoleucine, tyrosine or phenylalanine, added after growth of strains on minimum urea medium, was comparable in a wildtype and *ssy1* strain, thus nonexpression of *AGP1* in an *ssy1* strain in not due to inducer exclusion (Iraqui et al., 1999a). Therefore, sensing of amino acids appears to occur outside the cell, and Ssy1 is a good candidate to act as this sensor. Ssy1 may function by activating a hypothetical signal transduction pathway in response to detection of external amino acids, leading ultimately to transcriptional activation of several permease genes.

1.3.3.3 Ptr3

Barnes et al. (1998) have isolated a third gene, *PTR3*, whose product is required for amino acid-inducible expression of *PTR2* and *BAP2*. *PTR3* encodes a hydrophilic protein with a small region containing a sequence similar to members of the AAP family of proteins. Although Ptr3 does not contain putative membrane spanning regions, it appears to be tightly associated with the plasma membrane on the cytosolic side, cofractionating with Ssy1, the plasma membrane ATPase, and an additional plasma membrane marker in subcellular fractionation experiments (Klasson et al., 1999). As single and double *ssy1 ptr3* mutants manifest indistinguishable phenotypes in amino acid transport and both are membrane-bound, they may interact, functioning as components of a sensor of extracellular amino acids. A model proposed by Klasson et al. (1999) suggests that the region of similarity between Ptr3 and AAP family members may function as part of an amino acid binding region, detecting intracellular amino acid

levels imported at regulatory, and as yet undetected, levels by Ssy1. Certain events, such as amino acid binding, may induce Ptr3 to disengage from the plasma membrane and localise to other regions of the cell, exerting a controlling function on other members of the amino acid inducible cascade.

A number of other proteins have also been identified that function in amino acid-mediated induction of amino acid permease genes. Whilst Stp1 did not bind with UAS_{aa}, an unidentified compound did form a complex. Complex formation correlated with UAS_{aa} activity, thus this factor may be required for activation of permease genes via UAS_{aa}, in response to amino acid addition (de Boer et al., 1998). The *BAP2* and *BAP3* promoters have putative binding sites for the transcriptional activators of amino acid biosynthetic genes, Leu3 and Gcn4. Transcriptional activation of these genes by leucine does not require Leu3 or Gcn4, however, as leucine induction was observed at a level similar to wildtype in *leu3* and *gcn4* mutant strains (Didion et al., 1996; de Boer et al., 1998). The *leu3* mutant had high, constant *BAP3* expression, independent of leucine presence, so Leu3 may act as a represser of leucine-dependent induction, in the absence of leucine (de Boer et al., 1998). Amino acid-mediated induction of *AGP1* requires Uga35 (Dal81/DurL), a Cys6-Zn^{2+}-type transcription factor required for function of some nitrogen catabolite pathways. Uga35-mediated transcriptional induction of other genes such as of *UGA4* in the presence of GABA, is unaltered in *ssy1* mutants, thus Uga35 does not specifically mediate transcriptional induction in response to Ssy1 activity. Induction of *AGP1* also requires Grr1, the F-box protein of the SCF^{Grr1} ubiquitin ligase complex, required for transduction of the glucose signal generated by Snf3 and Rgt2 glucose sensors (Iraqui et al., 1999a). Mutations in *SSY3* and *SSY5* also exhibit similar phenotypes to mutations in *SSY1*, *STP1* and *PTR3*. Moreover, these mutations abolish increases in amino acid uptake resulting from a truncation of the C-terminal end of Bap2. Ssy3 and Ssy5 may therefore also have a regulatory function over *BAP2* expression, and may be components of the amino acid induction regulatory cascade (Jørgensen et al., 1998). Additional research is therefore required to elucidate the roles of Uga35, Grr1, Ssy3 and Ssy5 in amino acid induction of permeases, such as which of these proteins, if any, functions as the unidentified, hypothetical transcriptional activator proposed by de Boer et al. (1998).

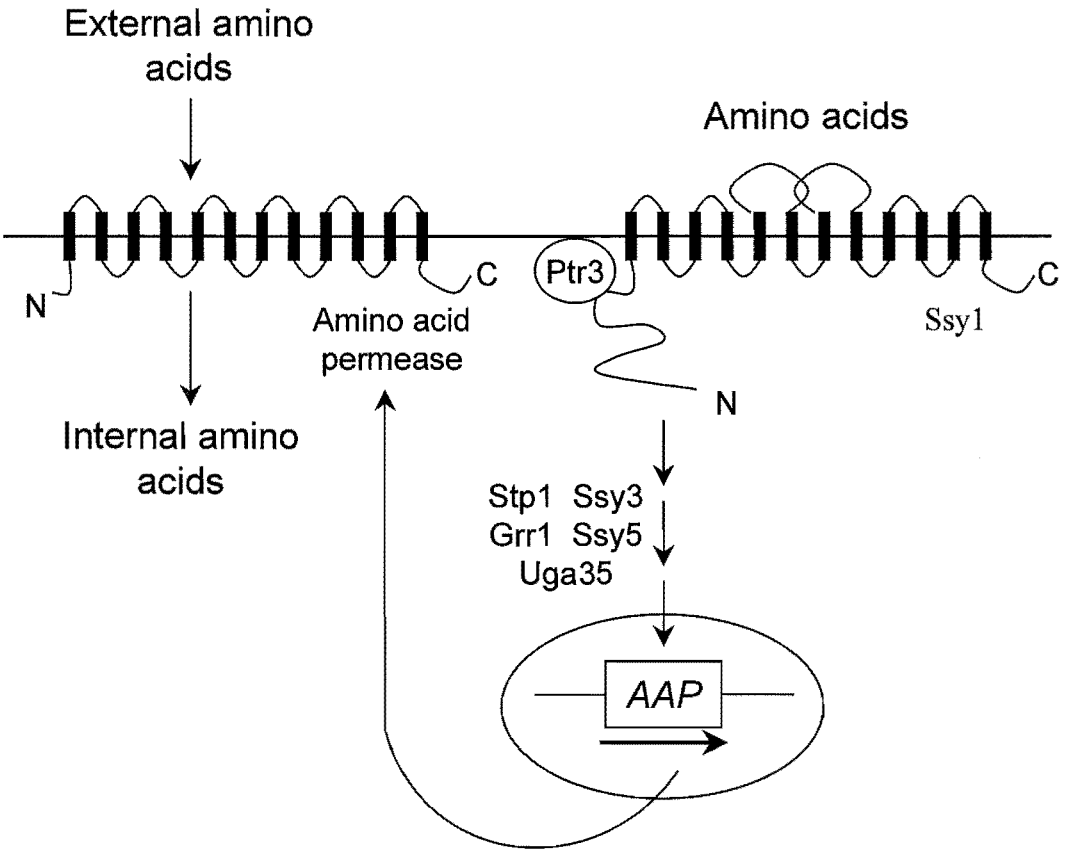

Figure 1.6: Diagram showing the role of the permease-like sensor Ssy1 in the transcriptional regulation of amino acid permease genes (*AAP*) sensitive to amino acid induction in *S. cerevisiae*. Extracellular amino acids are detected by extended external inter-transmembrane regions, and the signal is relayed by a cascade involving Ptr3, Stp1 and perhaps Ssy3, Ssy5, Grr1 and Uga35, ultimately resulting in induction of *AAP* transcription. Ptr3 is depicted as bound to Ssy1, however this is hypothetical. Adapted from Iraqui et al., 1999.

The expression of at least six genes encoding yeast amino acid and peptide permeases is therefore induced by the addition of amino acids to growth media, by an apparently common regulatory cascade, initially involving a plasma membrane Ssy1/Ptr3 system sensing external amino acids (Figure 1.6). One is tempted to draw an analogy between this pleiotropic regulatory system in yeast and a global regulator of metabolism in *E. coli*, the leucine-responsive regulatory protein (Lrp1). Lrp1 influences expression of operons involved in dipeptide transport and branched-chain amino acid transport, biosynthesis, and degradation, often in response to elevated levels of leucine in the environment. In general, anabolic target genes are positively regulated and catabolic genes are negatively regulated by Lrp1 (Calvo and Matthews, 1994). Yeast amino acid permeases that are ideally suited for providing amino acids for a catabolic role have

been described previously as those that are negatively regulated by good quality nitrogen sources. In keeping with the Lrp1 regulation analogy, nitrogen-regulated *GAP1* is not induced by presence of amino acids, and Klasson et al. (1999) have reported four- to 15-fold higher levels of *GAP1* mRNA in *ssy1* or *ptr3* mutants. Conversely, excluding *AGP1*, all other genes reported to be positively regulated by the Ssy1/Ptr3 system are not highly negatively regulated by readily utilisable nitrogen sources. Indeed, Tat2 is positively regulated by these nitrogen sources. Thus, most genes regulated by the Ssy1/Ptr3 system encode permeases that are well suited to providing amino acids for anabolic roles.

1.3.4 Feedback Inhibition

While some amino acids induce their own uptake at lower concentrations, for most amino acid permeases, preloading of yeast cells with an amino acid decreases further uptake of that same amino acid, and the degree of inhibition increases with increasing intracellular amino acid concentration. This ability of intracellular amino acids to inhibit influx of the same, and sometimes different amino acids, is feedback inhibition. Feedback inhibition has been observed in almost every yeast amino acid permease system tested so far, for example, inhibition of the histidine permease, Hip1, by histidine (Crabeel and Grenson, 1970); Gap1 by any amino acid transported by Gap1 (Grenson, 1992), the lysine permease by lysine (Morrison and Lichstein, 1976), methionine permease by methionine, and a threonine permease by threonine and serine (Gits and Grenson, 1969).

The mechanism or mechanisms underlying feedback inhibition remain unclear. Amino acid transport appears to be a unidirectional process as efflux from cells is either extremely low or undetectable (Cooper, 1982; Eddy, 1982). Decreases in rates of amino acid uptake are therefore not due to increased efflux of accumulated substrate. Kinetic studies have indicated that feedback inhibition affects the V_{max} of transport systems, while leaving the K_m intact (Grenson, 1992). This process must therefore either reduce the function, or the amount of the permease. Systems for transport of amino acids in *N. crassa* are also subject to feedback inhibition. This inhibition is rapid and not influenced by the presence of the protein synthesis inhibitors cycloheximide and blastocidine S. Inhibition is therefore not due to repression of formation of new

permease molecules and the dilution of existing permease activity, although this may also be occurring (Horák, 1986). Indeed, *BAP2* and *BAP3* promoters contain sequences that resemble the consensus target for Leu3 (Didion et al., 1996; de Boer et al., 1998), a protein involved in the repression of several leucine biosynthetic genes in response to leucine. Evidence exists that Leu3 negatively regulates *BAP3* expression (de Boer et al., 1998), and therefore may function as a transcriptional represser of both *BAP2* and *BAP3* when internal leucine concentrations get too high. A model proposed to explain the feedback inhibition phenomenon assumes that the amino acid permease does not change conformation to return the carrier to the external surface, when the carrier is still occupied by an amino acid. Therefore, when intracellular levels of amino acid are high, the amino acid may still occupy the permease carrier. Preloading of cells with amino acids not transported by the Hip1 permease does not affect histidine uptake (Crabeel and Grenson, 1970), and the latter model is consistent with substrate and inhibitor specificity being the same. It has not been determined whether the same binding site is used to transport solutes into the cell and to mediate feedback inhibition, though. Also, according to this model, the degree of inhibition would be correlated with how fast amino acids are compartmentalised into the vacuole. Against this theory, however, glycine transport into starved yeast suspensions was scarcely diminished when cells had absorbed the same amount of glycine required to inhibit uptake in unstarved cells, and accumulation continued until cells burst (Eddy, 1982). Therefore, feedback inhibition in this instance may not necessarily be dependent on the permease itself. Whether permease inactivation by feedback inhibition is reversible upon depletion of intracellular amino acid pools or requires *de novo* permease synthesis also remains to be determined.

1.4 THE LUP SYSTEM

The previous sections have presented an overview of yeast amino acid permease structure and function. Particular focus has centred on the specificity, capacity and affinity of permeases that transport hydrophobic amino acids, and the processes by which these are regulated according to the nitrogen source. Heinemann et al. (1994) have proposed that an additional hydrophobic amino acid-specific permease exists, capable of high affinity and low velocity transport. In the presence of ammonium, this

system was negatively regulated by an unidentified mechanism, and mutants that displayed deregulated transport were easily identified. We are interested in the identity of this permease, the mechanism by which it is regulated in the presence of ammonium and why mutants displaying deregulated transport arise at a high frequency.

Heinemann et al. (1994) observed a phenomenon whereby phenotypically and genotypically Leu$^-$ (*leu2-3, 112*) cells could give rise to small colonies on media containing insufficient concentrations of leucine to support the growth of the progenitor strain. Variants remained unable to grow when transferred to media devoid of leucine, thus reversion of the *leu2-3, 112* allele had not occurred. The growth phenotype of variants was genetically stable and inherited in a recessive manner, unlinked to *LEU2*. The ability to grow on concentrations of leucine that limited the wildtype (*leu2*) implied that mutants were better able to scavenge leucine from the environment. The mutant phenotype was therefore designated Lup$^+$ (Leucine Uptake), resulting from a mutation in the *LUP1* gene. Whilst Lup$^+$ variants were advantaged over Lup$^-$ strains on leucine gradient plates, growth characteristics of mutant and wildtype strains were indistinguishable on histidine or uracil gradient plates. [^{14}C]leucine uptake studies confirmed that mutant variants were better than the progenitor at transporting leucine. Leucine-starved Lup$^+$ variants transported 10 to 20 times more leucine than did the similarly leucine-starved wildtype, when leucine was supplied at concentrations between 0.3125-10 mM. Uptake only differed between strains when cells were grown in media containing ammonium as a nitrogen source. In minimal media containing proline as a nitrogen source, leucine was transported by mutant and wildtype strains at a similarly high rate, which was roughly equivalent to transport rates into Lup$^+$ strains when grown in MA medium. Kinetic parameters of leucine uptake by Lup$^+$ strains consisted of a V_{max} of 4570 pM leucine per 10^7 cells per 15 min, and a K_m of 3.7 μM. In similar uptake studies, Heinemann et al. (1994) compared the inhibition of [^{14}C]leucine transport by 100-fold higher concentrations of competitor amino acids into Lup$^+$ and Lup$^-$ strains, grown in nitrogen-repressing or nonrepressing conditions. [^{14}C]Leucine transport into the mutant was strongly inhibited by the hydrophobic amino acids leucine (95%), methionine (78%), isoleucine (87%), phenylalanine (93%), valine (64%), and tryptophan (31%), but not at all by histidine, lysine, threonine, or serine. Strong inhibition of leucine uptake by the hydrophobic amino acids suggested that these

were also transported by the system responsible for leucine transport in the Lup^+ strain. [^{14}C]-labelled phenylalanine and methionine uptake into Lup^+ and Lup^- strains, grown under nitrogen-repressing conditions, was therefore compared. As predicted, uptake of these amino acids into the mutant was considerably higher than into the wildtype. The extremely similar rates of leucine uptake, and profiles of amino acid inhibition of this uptake, between Lup^+ mutants grown in both MA and MP media and wildtype strains grown in MP medium, led them to propose that the same hydrophobic amino acid uptake transport system is present in both strains, and that that system is derepressed in the presence of ammonium in the mutant. The recessive nature of the mutant phenotype is consistent with inactivation of a represser of the high affinity uptake system, rather than a mutation in the permease itself, rendering it insensitive to negative regulation. Lup1 was therefore proposed to be a regulatory component of the high affinity, low velocity hydrophobic amino acid uptake pathway, and represses this system in the presence of ammonium (Figure 1.7).

The putative permease regulated by Lup1, referred to in this study as the Lup permease, appears to differ from previously characterised hydrophobic amino acid transport permeases. The ammonium repressibility of this system led researchers to investigate whether the Lup permease was the Gap1 permease, deregulated in the mutant strain. Gap1 could be distinguished from Lup, however. Gap1 has a higher affinity for basic amino acids, such as histidine and lysine, which had no effect on leucine transport into Lup^+ or Lup^- strains grown in MA medium, and the mutant was not advantaged on histidine gradient plates, contrary to expectations for Gap1. Also, transport of [^{14}C]citrulline, which only occurs via Gap1, remained regulated by ammonium in both the wildtype and mutant. When strains were grown in MA, the absolute amount of citrulline uptake was the same in the mutant and wildtype. Moreover, the low K_m for leucine transport by the Lup permease indicated that this system has a high affinity for leucine, whereas the Gap1 permease has a low affinity.

The Lup permease also appears to differ from Bap2. Although Bap2 also transports leucine in a high affinity, low velocity manner, the K_m values for this permease are 10-fold higher than those reported for the Lup permease, suggesting that the Lup permease has an even higher affinity for leucine. It remains possible that discrepancies are due to differences in strain backgrounds in which kinetic parameters were measured.

Nevertheless, substrate specificities of the Bap2 and Lup permeases also differ. Bap2 has more stringent substrate specificity, and while it transports leucine, valine and isoleucine, it does not transport phenylalanine and methionine. Like the Lup permease, Kotliar et al. (1994) have reported that Bap2 permease function is sensitive to ammonium repression, however this is not occurring at the level of NCR (Didion et al., 1996). Increased uptake from the Bap2 permease results from truncation of the C-terminal portion of Bap2 (Grauslund et al., 1995), the region containing the conserved DXKSS motif required for ubiquitination and subsequent degradation of permeases sensitive to NCI regulation, consistent with regulation of the Bap2 permease by this process.

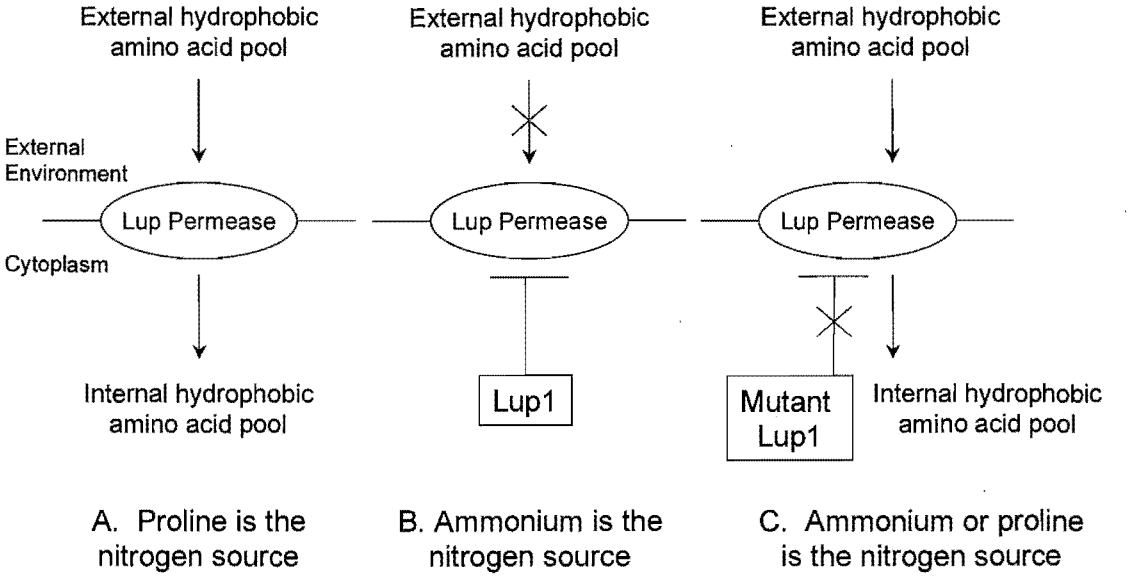

Figure 1.7: Proposed regulation of hydrophobic amino acid Lup permease by Lup1. Lup1 negatively regulates the permease in the presence of ammonium but not proline, or in the Lup$^+$ mutant. Lup1 is depicted as imposing its negative function directly on the permease, however, whether Lup1 acts directly or indirectly, and at the transcription or post-transcriptional level, remains to be determined. Arrows represent positive regulation and bar-ended lines depict negative regulation.

Of the other yeast amino acid permeases described as transporting leucine and other hydrophobic amino acids, S2, Tat1, Tat2, Agp1, Mup1 and Mup3, only Agp1 is strongly negatively regulated by the presence of good nitrogen sources, whilst S2 is also negatively regulated to some extent, and Tat2 is positively regulated under these conditions. Substrate affinities of Agp1 and S2 also most closely resemble Lup permease profiles, although unlike the Lup permease, Agp1 and S2 permeases also transport serine and threonine, and in addition, the Agp1 permease transports histidine. Affinities for leucine by all of the above systems are medium to low also, thus none of these systems appear to be equivalent to the Lup permease. It is possible, however, that a combination of permeases that transport leucine in addition to hydrophobic amino acids may be regulated by Lup1. Hydrophobic amino acids may be competing with leucine for uptake via various permeases, rather than a single pathway. Given the similarity of known amino acid permeases to one another, and the pleiotropic effects on many permeases by single mutations in permease regulators, it is not surprising that Lup1 may be regulating additional permeases. If more than one permease is contributing to leucine uptake over the [^{14}C]leucine concentrations Heinemann et al. (1994) carried out uptake studies in, this could be revealed by non-linear curves in plots of kinetic data such as the Lineweaver-Burk plot.

Inhibition profiles for the Lup permease resemble results obtained for an *aat1* mutant by Garrett (1989). Strains containing a combination of the *aat1* and *leu2* alleles were unable to grow on rich media or on MA or MP media supplemented with isoleucine, methionine, phenylalanine, tyrosine or valine. Serine and threonine also partially inhibited growth when present at higher concentrations. Uptake of leucine was inhibited to less than 35% of wildtype levels in *aat1* mutants preincubated with the amino acids that inhibit growth. Garrett (1989) therefore proposed that Aat1 is required for leucine transport in the presence of these amino acids. Heinemann et al. (1994) have suggested that Aat1 may be required for the function of a leucine permease distinct from the one that Lup1 purportedly regulates. In the absence of Aat1, leucine uptake is dependent upon alternative permeases, and subject to competitive inhibition by other substrates of these permeases. Accordingly, leucine transport via the Lup permease would be inhibited by hydrophobic amino acids, and when the *aat1* mutation was accompanied by the *leu2* allele, leucine deprivation caused by inhibited uptake resulted in growth retardation. Inhibition of leucine uptake by these amino acids was decreased

when yeast was grown on MA medium (Garrett, 1989), consistent with the contribution by the ammonium-regulated Lup permease to hydrophobic amino acid-inhibited leucine uptake in *aat1* variants. Overexpression of *BAP2* provides cells with additional leucine-transporting permeases and, consequently, relieves the *aat1* YPD$^-$ defect (Schreve and Garrett, 1997). Whether Bap2 comprises the permease regulated by Aat1 remains undetermined.

As Lup1 appears to negatively regulate the Lup permease in response to nitrogen, Lup1 may play an analogous role in NCR or NCI to one of the negative regulators Ure2, Dal80, Deh1, Rsp5, Doa4/Npi2, Per1 or Aua1. It is unlikely that Lup1 actually comprises one of these proteins, however, as all are regulators of the Gap1 permease, and this system remains negatively regulated in the presence of ammonium in the *lup1* mutant. However, as regulation of Gap1 is mediated by dual systems in response to nitrogen, it is possible a defect in one system is masked by the other system.

Other mutations in genes affecting amino acid transport including *apf1/aap1/shr3*, *raa1*, *raa2*, *raa3* and *raa4*, also do not appear to be related to *lup1*. Mutations in the allelic genes *AAP1* (Surdin et al., 1965), *APF1* (Grenson and Hennaut, 1971) and *SHR3* (Ljungdahl et al., 1992), confer resistance to toxic amino acid analogs and toxic concentrations of L-histidine. Other characteristics of mutants include an inability to grow on proline as a nitrogen source, repressed Gap1 activity in all media, and greatly decreased transport of a wide variety of amino acids. Whilst amino acid accumulation was decreased in *apf1/aap1/shr3* mutants, proton-ATPase activity, membrane potential, and transport by other proton-driven symports was unaffected (Horák and Kotyk, 1993). Apf1/Aap1/Shr3 was found to be an integral protein of the endoplasmic reticulum and is required for processing and translocation of amino acid transport permeases from the endoplasmic reticulum to the plasma membrane (Ljungdahl et al., 1992; Horák and Kotyk, 1993). Like *apf1/aap1/shr3* mutants, strains containing the *raa1, 2, 3* and *4* mutations are resistant to amino acid analogs. Resistance is due to reduced uptake of analogs and occurs in urea, but not proline, media (McCusker and Haber, 1990). These mutations are not allelic to *GAP1* mutations as *raa* mutants can use citrulline as a nitrogen source. *raa* mutations are also not related to *apf1/aap1/shr3* mutations as diploids created from both mutants were able to grow on proline, so the two recessive mutations were not in the same gene. Therefore, unlike Lup1, Apf1/Aap1/Shr3

positively regulates permeases, and decreased amino acid transport in *raa* mutants suggests that Raa1, 2, 3 and 4 are, likewise, positive regulators.

Lup^+ mutants have been observed to occur at a high frequency. After 2 to 4 days of growth on plates containing concentrations of leucine limiting to the progenitor, 1 in 4 x $10^6 \pm 1$ x10^6 input cells had acquired this phenotype. Moreover, the phenotype was eventually displayed by a large percentage of the population (1 variant in every 100 input cells after 12 days) (Heinemann et al., 1994). Why mutations in *LUP1* appear to arise so frequently is unclear. This gene may contain hotspots for mutation such as by transposon insertion. Only a couple of mutants were characterised further, and these have similar phenotypes, thus may have mutations in the same gene. No attempt has been made to rule out the assumption that all mutants occurring also have a mutation in this same gene. Lup1 may comprise a component in a multicomponent cascade regulating the Lup permease. Given the multiplicity of permeases capable of transporting leucine, and the plethora of proteins regulating these, it is reasonable to predict that a mutation in one of a number of genes encoding these permeases or regulators may result in cells able to grow on lower leucine concentrations. Calculations by Heinemann et al. (1994) of the mutation rate of wildtype cells to the Lup^+ phenotype assumed that continued DNA metabolism and growth of wildtype cells was not occurring on selection plates, however no attempts were made to rule this out. In all likelihood, cell turnover was still occurring on selection plates. Indeed, the wildtype strain incubated in liquid media containing 0.01 mg leucine mL^{-1} (the concentration of leucine used in plates selecting for mutants), had a 23 hour generation time. When the concentration of leucine was doubled, the generation time halved. Leucine could have been released from deceased yeast on selective plates providing leucine to starved cells, and in light of the latter results, this small leucine supplement may have a significant effect on the number of generations undergone on selection plates (Heinemann et al., 1994).

1.5 RESEARCH APPROACH

The primary objectives of this study were to further characterise the Lup system, to provide information regarding regulation of the Lup permease by identification of Lup1,

and to investigate the nature of the high mutation rate to the Lup^+ phenotype. The following approaches were taken to address these objectives:

1. A positive selection was established for strains heterozygous at the *LUP1* locus based on the predicted propensity of Lup^+ mutants to better accumulate toxic analogs of hydrophobic amino acids, for use in cloning as well as providing information on the specificity of the Lup permease.

2. Genes that complemented the mutant *lup1* allele were isolated from wildtype libraries using the positive selection strategy developed in (1.).

3. Transposon mutagenesis and subcloning was used to narrow down the boundaries of complementing genes within complementing plasmids.

4. The DNA sequence of the region encompassing the complementing gene was established and compared with known DNA sequences stored in databases.

5. The complementing gene was confirmed to be allelic to *LUP1* by:

   i. Construction of a strain containing a partially deleted chromosomal copy of the cloned, putative *LUP1* allele, and comparison of phenotypes of this strain with those of a characterised *lup1* mutant.

   ii. Creation of diploid strains by mating a characterised *lup1* strain with a strain containing a chromosomal null allele of cloned, putative *LUP1*, followed by analysis of phenotypes of diploids and their offspring following sporulation.

6. The *lup1* sequence was amplified by PCR using primers determined from the *LUP1* sequence. *LUP1* and *lup1* sequences from several mutants were compared to determine the nature of the mutation.

7. A model of regulation of the putative Lup permease by Lup1 was constructed from information gained, and predictions of this model were tested.

2. MATERIALS AND METHODS

2.1 BACTERIAL AND YEAST STRAINS, PLASMIDS, TRANSPOSONS AND PHAGES

Bacterial and yeast strains, plasmids, transposons and phages used in this study are listed in Table 2.1.

Table 2.1: Strains, plasmids, transposons and phages related to this study.

Strain or Plasmid	Genotype or Description	Reference or Source
E. coli		
DH10B	*hsdR hsdM rpsL deoR* ϕ80d*lacZDM15 endA1 recA1 mcrA* Δ(*mrr hsdRMS*) *mcrBC*	Grant et al., 1990
MC4100	*supO rpsL150 relA1 deoC1*	J. Heinemann collection
PB2480	*lacY1 supE44 ponA21*	H. K. Mahanty collection
S. cerevisiae		
329-6C	*MAT*α *his3-537::TRP1::his3-513 leu2-3,112 trp1 ura3-52 ade6*	Klein, 1988
JY117	spontaneous Lup$^+$ mutant of 329-6C	Heinemann et al., 1994
SY1229	*MAT***a** *leu2-3 leu2-112 ura3 his3*	Heinemann and Sprague, 1989
JY107	SY1229 background, *URA3*	Heinemann et al., 1994
JY126	JY117 background, *HIS3*	Heinemann et al., 1994
JY127	JY107 × JY126	Heinemann et al., 1994
JOY53	spontaneous Lup$^+$ mutant of 329-6C	This study
JOY62, 63, 80-88	329-6C containing integrated 2.2 kb *Sac*I/*Cla*I fragment from pJO60, Lup$^-$	This study
JOY64-68	329-6C containing 2.2 kb *Sac*I/*Cla*I fragment from pJO60 integrated at *BUL1*, Lup$^+$	This study
JOY73	spontaneous His$^+$ mutant of JY117	This study
JOY74	SY1229 containing 2.2 kb *Sac*I/*Cla*I fragment from pJO60 integrated at *BUL1*, Lup$^+$	This study
JOY77	JOY73 × JOY74	This study

Table 2.1: continued

Strain or Plasmid	Genotype or Description	Reference or Source
YHY007K	KA31-2A background, *bul2::URA3*	Yashiroda et al., 1998
KA31-2A	*MATa his3 leu2 ura3 trp1*	Yashiroda et al., 1998
YHY008K	YHY007K background, *bul2::URA3*	Yashiroda et al., 1998
YAT2-1C	*MATα his3 leu2 trp1 rsp5-101 ura3* A daughter resulting from sporulation of diploid created from a cross between KA31-2A and a nonisogenic *rsp5-101* mutant	Yashiroda et al., 1996
***E. coli* Plasmids**		
pBluescript® SK(+/-)	*lacZ* Ap^R	H. K. Mahanty collection
pJO45	pBluescript® SK(+/-) containing 0.45 kb *Bam*HI fragment from pJO34, Lac^-	This study
pJO46	pBluescript® SK(+/-) containing 1.5 kb *Bam*HI fragment from pJO38, Lac^-	This study
pJO47	pBluescript® SK(+/-) containing 1.0 kb *Bam*HI fragment from pJO39, Lac^-	This study
pJO58	pBluescript® SK(+/-) containing 1.45 kb *ClaI*/*Sac*I fragment from pJO13, Lac^-	This study
pJO59	pJO58 with 0.43 kb *Eco*RV fragment deleted	This study
pJO60	pJO59 containing 1.17 kb *Hin*dIII *URA3* fragment YEp24	This study
***E. coli* and *S. cerevisiae* Plasmids**		
YEp24	Ap^R, Tc^R, *URA3*	J. Heinemann collection
YCp50	Ap^R, Tc^R, *URA3*	J. Heinemann collection
pJO13	Fpa^R–conferring Rose library plasmid, Ap^R, *BUL1*	This study
pJO14	Fpa^R–conferring Rose library plasmid, Ap^R, *BUL1*	This study
pJO15	Fpa^R–conferring Rose library plasmid, Ap^R, *BUL1*	This study
pJO16	Fpa^R–conferring Rose library plasmid, Ap^R, *BUL1*	This study

Table 2.1: continued

Strain or Plasmid	Genotype or Description	Reference or Source
pJO17	Fpa^R–conferring Botstein library plasmid, Ap^R, *BUL1*	This study
pJO18	Fpa^R–conferring Botstein library plasmid, Ap^R, *ARO4*	This study
pJO20	Fpa^R–conferring Botstein library plasmid, Ap^R, *ARO4*	This study
pJO21	Fpa^R–conferring Botstein library plasmid, Ap^R, *ARO4*	This study
pJO22	Fpa^R–conferring Botstein library plasmid, Ap^R, *ARO4*	This study
pJO23	Fpa^R–conferring Botstein library plasmid, Ap^R, *ARO4*	This study
pJO24	Fpa^R–conferring Botstein library plasmid, Ap^R, *BUL1*	This study
pJO25	Fpa^R–conferring Rose library plasmid, Ap^R, *BUL1*	This study
pJO26	Rose library plasmid from spontaneous Lup^+ mutant, Ap^R	This study
pJO27	Fpa^R–conferring Botstein library plasmid, Ap^R, *BUL1*	This study
pJO28	Fpa^R–conferring Botstein library plasmid, Ap^R, *ARO4*	This study
pJO31	YCp50 containing 2.3 kb *Eco*RI/*Bam*HI fragment from pJO13, Tc^S	This study
pJO32	YCp50 containing 3.6 kb *Eco*RI/*Hin*dIII fragment from pJO13, Tc^S	This study
pJO33	pJO24 containing *Tn*10 derivative 103, Lup^+	This study
pJO34	pJO24 containing *Tn*10 derivative 103, Lup^+	This study
pJO38	pJO21 containing *Tn*10 derivative 103, Fpa^S	This study
pJO39	pJO21 containing *Tn*10 derivative 103, Fpa^S	This study
pJO48	pJO24 with 2.6 kb *Sac*I fragment deleted	This study
pHY37	*CEN6 ARSH4 URA3 bul1*$^{P157Q, P158A}$	Yashiroda et al., 1998
pHY32	*CEN6 ARSH4 URA3 BUL2*	Yashiroda et al., 1998

Table 2.1: continued

Strain or Plasmid	Genotype or Description	Reference or Source
pHY08	*CEN4 ARS1 URA3 RSP5*	Yashiroda et al., 1996
***E. coli* Phage**		
λNK1316	contains Tn*10* derivative 103	Kleckner et al., 1991
Transposon		
mini-*Tn*10 derivative 103	*kan*/P*tac*, ATS transposase	Kleckner et al., 1991

2.1.1 Yeast Genomic DNA Libraries

The Rose library was constructed by Rose et al. (1987). Chromosomal DNA from *S. cerevisiae* GRF88 (MATa *his4*-38, S288C background) was partially digested with *Sau*3A to create 10-30 kb fragments, which were inserted into the *Bam*HI site of YCp50. The Botstein library was created from the insertion of *S. cerevisiae* DBY939 (*suc2-215am*, S288C background) chromosomal DNA fragments, also arising from partial *Sau*3A digestion, into the *Bam*HI site of the YEp24 vector (Carlson and Botstein, 1982).

Libraries were acquired as isopropanol precipitates, and were amplified in *E. coli* DH10B. Transformants were pooled into six pools, each consisting of 3000 to 4000 transformant colonies. DNA was prepared from large-scale cultures, and stored as isopropanol precipitates at –20°C.

2.2 MEDIA AND GROWTH CONDITIONS

2.2.1 Growth Media and Supplements

All media was prepared as described in Appendix 1. When solid media was required, agar was added to a concentration of 1.5% (w/v), and LB Top agar for overlays was

solidified with 0.7% (w/v) of agar. Minimal media and agar were autoclaved separately, and added together following autoclaving.

A number of variations of Synthetic Dextrose (SD) medium were utilised. SD medium containing all amino acids and supplements for yeast minimal media described in Appendix 1, was designated Synthetic Complete (SC) medium. Various combinations of the supplements leucine (LEU), histidine (HIS) uracil (URA), tryptophan (TRP) and adenine (ADE) were added to SD at the concentrations specified in Appendix 1. SC medium with one or more of these amino acids omitted was also used. SD medium used for Plate Gradient Assays (PGAs) and Minimum Inhibitory Concentration (MIC) determinations contained one half the concentration of histidine, uracil, leucine and adenine concentrations specified in Appendix 1, and was designated SD + 0.5 HULA medium. Sometimes tryptophan was also incorporated into SD + 0.5 HULA medium at one half of the concentration specified in Appendix 1, to create SD + 0.5 HULAT. Limited Leucine medium (LLM) consisted of SC - LEU supplemented with 2.5 mg L^{-1} of leucine.

Antibiotics were added to media at concentrations specified in Appendix 1. Antibiotics were stored as 1000x stock solutions at –20°C. Ampicillin, kanamycin and streptomycin were dissolved in dH_2O and filter sterilised through 0.22 μm pore Millipore filters, and tetracycline was dissolved in methanol. When added to molten media, media was cooled to 50°C before antibiotic addition.

To supply 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) to media, 40 μl of a 20 mg mL^{-1} stock solution, dissolved in dimethyl formamide, was spread on plates and allowed to dry for 30 min prior to inoculation of plates. When required, 4 μL of isopropyl-β-thio-galactopyranoside (IPTG, 200 mg mL^{-1}) was spread on plates in conjunction with X-gal.

2.2.2 Growth Conditions

Unless specified, *E. coli* and *S. cerevisiae* cultures were incubated at 37°C and 30°C, respectively. When grown in liquid media, cultures were incubated in Gyrotory® Water

Bath Shakers (New Brunswick, G76D), shaken at 250 rpm. Saturated cultures were obtained by inoculating a single colony into 10 mL of LB (bacteria), or YPD (yeast), in 25 mL Universal bottles, and incubation for 15-18 hr (overnight). After overnight growth in rich media, *E. coli* numbers typically reached 1 x 10^{10} cfu mL^{-1}, and *S. cerevisiae* numbers reached 2 x 10^{8} cfu mL^{-1}. When inoculated onto solid LB, *E. coli* colonies generally took less than 24 hr to appear, compared to up to 48 hr on minimal media. *S. cerevisiae* colonies typically appeared after two days incubation on solid YPD plates, and three days when plated onto minimal media.

2.2.3 Storage of Bacteria and Yeast

For permanent storage, 1 mL of a saturated culture of *E. coli* or *S. cerevisiae* was pelleted by centrifugation in a microfuge. Cells were resuspended in 1 mL of sterile 15% glycerol, and stored at –80°C. For short-term storage, strain stocks were streaked from –80°C stocks onto appropriate agar plates and stored for two weeks at 4°C.

2.3 ASSESSMENT OF TOXIC AMINO ACID ANALOG SENSITIVITY

The sensitivity of various strains to toxic amino acid analogs was evaluated using Plate Gradient Assays (PGAs) and Minimum Inhibitory Concentration (MIC) determination.

2.3.1 Plate Gradient Assays (PGAs)

PGAs were used to qualitatively compare sensitivity of strains to toxic analogs.

Aliquots of analog solutions were added to a hole with a 8 mm diameter created in the centre of agar plates, and allowed to diffuse through the medium, effectively creating a concentration gradient radiating out from the plate centre. After analog aliquots had dried, 10-fold dilutions of saturated cultures were applied to the plate as spokes extending outwards from the plate centre. After two to three days of incubation at 30°C, the distance from the centre hole in which analog was applied, to where growth of strains initiated, was measured. Distances were expressed as ratios of variant:wildtype.

2.3.2 Minimum Inhibitory Concentration (MIC) Determination

The level of analog inhibitory to various strains was determined quantitatively using MIC determination.

Analog was incorporated into medium at varying concentrations and plates were inoculated with 10^2 or 10^4 cells. MICs were defined as the lowest concentration of analog at which visible colonies were no longer apparent after three days of growth.

2.4 DNA MANIPULATIONS

All buffers and solutions required for DNA manipulation protocols are listed in Appendix 2.

2.4.1 Alkaline Lysis Plasmid Preparation from *E. coli*

Plasmid DNA of the quality required for restriction endonuclease digestion, ligation and transformation was prepared from *E. coli* using a procedure based on the Alkaline Lysis Procedure (Sambrook et al., 1989).

Cells from 1.5 mL from a saturated culture of *E. coli* grown in LB were harvested by centrifugation. The cell pellet was resuspended in 100 µL of Solution 1, and incubated on ice for 5 min. A volume of 200 µL of Solution 2 was added, and the tube contents were mixed well until viscous and clear. After incubation on ice for 5 min, 150 µL of Solution 3 was added. Tube contents were mixed gently until a white precipitate formed, followed by incubation on ice for 10 min. The precipitate was pelleted by centrifugation at top speed for 10 min, and the supernatant was recovered. For crude preparations of plasmid DNA, DNA was precipitated from the supernatant by the addition of 2 volumes of ice-cold, 100% ethanol, mixed, and incubated on ice for 10 min. Following centrifugation at top speed at 4°C for 10 min, the supernatant was aspirated. The DNA pellet was washed with 1 mL of 70% ethanol and this was removed after a 1 min centrifugation. The air-dried DNA pellet was resuspended in 20-

30 µL of $T_{10}E_1$ or dH_2O and stored at 4°C or –20°C. If a purer quality of plasmid DNA was required, remaining protein was extracted from the DNA-containing supernatant by phenol-chloroform extraction. DNA was precipitated from the recovered aqueous phase, washed with 70% ethanol, and resuspended in $T_{10}E_1$ or dH_2O, as mentioned previously.

2.4.2 Lithium Chloride Plasmid Preparation from *E. coli*

Plasmid DNA required for sequencing using the LI-COR Automated DNA Sequencer was prepared using the Lithium Chloride Plasmid Preparation Procedure. The procedure was adapted from the Alkaline Lysis Plasmid Preparation. An important modification included the addition of 5 M lithium chloride during alkaline lysis to remove high molecular weight RNA (Sambrook et al., 1989).

A 50 mL saturated culture was harvested by centrifugation at 5 000 *x g* for 2 min. The cell pellet was resuspended in 3 mL of Solution 1, and incubated on ice for 5 min. A volume of 6 mL of Solution 2 was added, and mixed until viscous and clear. Following incubation for 5 min at RT, 3 mL of Solution 3 was mixed in until a white precipitate formed. After a 10 min incubation on ice, the precipitate was pelleted by centrifugation at 13 000 *x g* for 10 min. A volume of 12 mL of isopropanol was added to the supernatant, and incubated for 10 min on ice. The pellet, recovered after centrifugation at 13 000 *x g* for 10 min, was air-dried and resuspended in 1 mL of $T_{10}E_1$. A volume of 1 mL of ice-cold 5 M lithium chloride was then added. Following incubation on ice for 15-30 min, tube contents were centrifuged at 13 000 *x g* for 10 min. The supernatant was recovered, from which nucleic acid was precipitated by the addition of 2 mL of isopropanol. After incubation on ice, the tube was centrifuged as previously, and the pellet recovered was air dried, dissolved in 200 µL and transferred to a microfuge tube. RNase was added at a final concentration of 10 µg mL^{-1} and incubated at 37°C for 15 min. The solution underwent phenol/chloroform extraction, and following ethanol precipitation, was dissolved in an appropriate volume of dH_2O or $T_{10}E_1$.

2.4.3 Plasmid Rescue from Yeast

Plasmid DNA was isolated from yeast for transformation of *E. coli* using a procedure adapted from Hoffman and Winston (1987).

1.5 mL of a YPD yeast culture, grown to saturation, was centrifuged in a microfuge at top speed for 15 sec. The pellet was resuspended in 0.2 mL of Buffer A, and 0.2 mL of phenol-chloroform-isoamyl (25:24:1) was added. Following addition of 0.2 mL of sterilised 0.45-0.52 mm glass beads, the cell mix was vortexed vigorously at top speed for 2 min. The aqueous layer was removed, mixed with an equal volume of chloroform-isoamyl (24:1), and centrifuged in a microfuge at top speed for 2 min. The aqueous layer was again recovered, and DNA present was precipitated using ethanol precipitation.

2.4.4 Yeast Chromosomal DNA Preparation

Chromosomal DNA was extracted from yeast using a variation of a protocol described by Nasmyth and Reed (1980).

A volume of 5 mL of cells from a saturated YPD culture of yeast was harvested by centrifugation at 9 000 *x g* for 2 min. The cell pellet was resuspended in 1 mL dH_2O, and repelleted by centrifugation. Cells were resuspended in 0.2 mL of SCEM and incubated at 37°C for at least 30 min, or until cell walls were degraded (observed by microscopy). STE was added to cells at a volume of 0.8 mL, and following gentle mixing, cells were incubated at 70°C for 20 min. After cooling on ice, 0.2 mL of 5 M potassium acetate was added, mixed in well, and incubated on ice for 45 min. The white precipitate that formed was sedimented by centrifugation for 10 min. The supernatant was recovered, and remaining protein was extracted by phenol/chloroform extraction. DNA from the aqueous phase was precipitated by addition of 2 volumes of ice-cold 100% ethanol and incubated on ice for 10 min. The DNA pellet resulting after centrifugation for 10 min at 4°C, was washed with 70% ethanol. All remaining ethanol was removed following a 1 min centrifugation, and the air-dried DNA pellet was resuspended in 30 to 50 µL of $T_{10}E_1$.

2.4.5 DNA Isolation from Agarose Gel Slices

DNA fragments to be used in ligations, knock-out construction or as probes in Southern analyses, or PCR products to be sequenced, were isolated from agarose gel slices using the Bio-Rad Prep-A-Gene® DNA Purification Kit, following the manufacturer's instructions.

The desired DNA band from an ethidium bromide-stained agarose gel was visualised using a handheld lamp (Mineralight® UVSL.25) set on longwave, excised and placed in a microfuge tube. The gel slice was weighed to estimate its volume in mL, assuming that 1 g of gel slice was equivalent to 1 mL. Based on the volume of the gel slice and the amount of Prep-A-Gene matrix to be added, 3 volumes of Prep-A-Gene DNA purification kit binding buffer was added to the gel slice, and incubated at 55°C for several min until the gel slice had dissolved. A volume of 5 µl of thoroughly resuspended Prep-A-Gene Matrix for every µg of DNA to be adsorbed was added, and incubated with frequent agitation for 10 min at RT. The DNA-bound Prep-A-Gene matrix was pelleted by centrifugation for 30 sec in a microfuge, and resuspended in a volume of DNA purification kit binding buffer equivalent to 25 times the volume of added matrix. The DNA-bound matrix was again centrifuged for 30 sec, and the resulting pellet was gently resuspended in 25 times the volume of added matrix of wash buffer. This step was repeated if DNA was to be eluted with $T_{10}E_1$. If sterile dH_2O was to be used for elution, 80% ethanol replaced wash buffer for this final wash. The DNA-bound matrix was pelleted firmly, and all traces of wash buffer were removed before the pellet was resuspended in at least 1 pellet volume of sterile dH_2O or $T_{10}E_1$. DNA was eluted from the matrix during a 5 min incubation at 45°C. Following a 1 min centrifugation, the DNA-containing supernatant was transferred to a clean tube, and centrifuged to check for, and remove if present, any remaining Prep-A-Gene matrix.

2.4.6 Electroporation-mediated Transformation of *E. coli*

DNA was introduced into *E. coli* cells using electroporation-mediated transformation, as described by Smith et al. (1990).

2.4.6.1 Preparation of Electrocompetant Cells

A fresh colony of the *E. coli* strain to be transformed was inoculated into SOB and grown to saturation at 37°C. A volume of 0.5 mL from the saturated culture was used to inoculate 500 mL of SOB, which was grown with vigorous aeration at 37°C until the OD_{600} had reached approximately 0.8 (about 2 to 3 hr). Cells were harvested by centrifugation at 4°C for 5 min at 2 600 *x g*. The resulting pellet was resuspended in 500 mL ice-cold WB, centrifuged, and resuspended in the same manner. Following centrifugation, cells were resuspended in WB to a final volume of 2 mL. Cells were frozen and stored in 0.2 mL aliquots at –80°C.

2.4.6.2 Electroporation

Frozen electrocompetant cells were thawed on ice prior to use. Plasmid DNA (up to 1 µg), dissolved in a volume of no more than 5 µL of $T_{10}E_1$ or water, was mixed with 40 µL of cells, and placed into a sterile, chilled, 0.2 cm gap micro-electroporation curvette. The curvette was pulsed once in a Bio-Rad Gene Pulser™ set to 2.5 kV at 25 µF and the Pulse Controller set to 200 Ω. Pulsed cells were quickly resuspended in 1 mL of SOB, transferred to a 1.5 mL eppendorf tube, and shaken at 37°C for 1 hr. Following elaboration, cells were diluted, plated on media to select for transformants, and incubated at 37°C.

2.4.7 Lithium Acetate-Mediated Yeast Transformation

Yeast cells were transformed using lithium acetate, as described by Gietz and Schiestl (1995).

A volume of 50 mL of YPD was inoculated with 5 mL from a saturated culture to a cell density of approximately 5 x 10^6 cfu mL^{-1}.. The culture was incubated with shaking for 3 to 5 h, until at least 2, and no more than 4, cell divisions had occurred. The culture was harvested by centrifugation at 3000 *g* for 5 min. Cells were washed in 25 mL of sterile dH_2O, and repelleted. The pellet was resuspended in 1.0 mL 100 mM sterile lithium acetate and recentrifuged at top speed for 15 min in a microfuge. Cells were resuspended to a final volume of 500 µL in 100 mM lithium acetate and 50 µL volumes were aliquoted into microfuge tubes for each transformation reaction. Following

centrifugation, the following solutions were added to the cell pellet in the order listed: 240 μL of PEG (50% w/v), 36 μL of 1.0 M lithium acetate, 25 μL of single stranded herring sperm carrier DNA (2.0 mg mL^{-1}) and plasmid DNA (0.1-10 μg) dissolved in 59 μL of sterile dH_2O. Tubes were vortexed until cell pellets had been mixed, and were incubated at 30°C for 30 min. Following a heat shock at 42°C for 20 to 25 min, cells were pelleted in a microfuge for 15 sec. The final cell pellet was gently resuspended in 1.0 mL sterile dH_2O. Volumes of 10 to 200 μL were plated onto media to select for transformants, and incubated at 30°C for 2 to 3 days to recover transformants.

2.4.8 Agarose Gel Electrophoresis

Agarose gels for the separation and visualisation of DNA fragments were composed of 0.7-1% agarose, depending on the fragment size to be visualised, dissolved in 0.5 x TBE. Gels were cast in a Mini-gel Hoefer HE47-10 Casting kit to create a 70 x 100 mm gel, using 8 or 12 well combs. When a larger gel was required, the gel was cast in a Bio-Rad gel mould sized 140 x 128 mm, using a 28 well comb. Prior to loading in prepared gels, DNA samples were mixed with 3 μL Loading Buffer. The electrophoresis running buffer consisted of 0.5 x TBE. Gels were typically run at 80 V. Gels were stained in a 0.5 μg mL^{-1} ethidium bromide solution for 20 min, and destained in dH_2O for 5 min, if required. DNA was visualised using an UV transilluminator (Ultraviolet products Ltd. Tm15), and results were photographed using a Polaroid MP4 Land camera with iso Polaroid black and white instant film. Sizes of restriction fragments were determined by comparing the distance of migration of known sized fragments to unknown sized fragments, using the computer program Dnafrag version 3.03 (National Research Council of Canada). Bacteriophage λ DNA, digested by *Hin*dIII, a 1 kb ladder (Gibco BRL) and a 100 base pair ladder (Gibco BRL) were used as markers of molecular weight.

2.4.9 Restriction Digestion of DNA

DNA was digested by restriction enzymes (Boehringer Mannheim) according to manufacturer's instructions. Typically, digestion was carried out in a total volume of 20 μL, for 2-4 hr, at 37°C. When double digestions were carried out, the most appropriate

buffer was chosen. When restriction digested DNA was required for agarose gel electrophoresis, the DNA was loaded directly in the gel. Other protocols, such as ligation and DNA dephosphorylation, required the removal of restriction enzymes, however. This was accomplished by denaturation of the enzyme by heat treatment at 65°C for 10 min, or by phenol/chloroform extraction, succeeded by ethanol precipitation.

2.4.10 Precipitation of DNA

Unless otherwise stated, DNA was precipitated from aqueous solutions as described by Sambrook et al. (1989).

A 0.1 volume of sodium acetate (3 M) was added to the DNA solution, followed by 2 volumes of ice-cold, 100% ethanol. Following mixing, the tube contents were incubated at 4°C or –20°C for 30 min. DNA was recovered by centrifugation for 10 min, at 20 000 *x g* and 4°C. The DNA pellet was washed with 1 mL of 70% ethanol, which was aspirated off following a 1 min centrifugation. The air-dried pellet was dissolved in an appropriate amount of $T_{10}E_1$ or dH_2O, and stored at –20°C or 4°C.

2.4.11 Phenol/Chloroform Extraction of Contaminants from DNA Solutions

Where specified, contaminants were separated from DNA-containing solutions using phenol/chloroform extraction, as described by Sambrook et al. (1989).

An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the aqueous solution requiring extraction. The solutions were mixed to a homogenous emulsion, and centrifuged for 2 min. The upper, aqueous phase was recovered, and an equal volume of chloroform/isoamyl alcohol (24:1) was added. The solutions were mixed and centrifuged as previously, and the upper, aqueous phase was again recovered.

2.4.12 Ligation of DNA Restriction Fragments

Ligation of DNA restriction fragments with overlapping ends was carried out according to manufacturer's instructions. Typically, ligations were carried out in a total volume of 20 μL, containing 4 μL of 5 x DNA Gibco BRL® Ligase Buffer, 1 μL of Gibco BRL® T4 DNA Ligase, and an insert DNA to vector DNA ratio of 3:1. Total DNA concentration was up to 0.1 μg for ligation of cohesive-ended fragments or up to 1 μg for ligation of blunt-ended fragments. Ligation of blunt-ended fragments was carried out for 24 hr at 14-16°C, and cohesive-ended fragment ligation was carried out at 24-28°C for 1 hr. Ligated DNA was ethanol-precipitated and dissolved in 5 μL of $T_{10}E_1$ or dH_2O prior to introduction into *E. coli*.

2.4.12 Dephosphorylation of Vector DNA

Prior to ligation, the 5' ends of restriction endonuclease-digested DNA vectors containing complementary ends, were dephosphorylated using Gibco BRL® Calf Intestinal Alkaline Phosphatase (CIAP) to eliminate self-ligation. Typically, reactions were carried out in a total volume of 20 μL, containing 2 μL of CIAP 10x Buffer and 1 unit of CIAP per μg of vector DNA present. Dephosphorylation was carried out at 37°C for 30 min. Following dephosphorylation, CIAP was inactivated by heating at 75°C for 10 min, or removed by phenol/chloroform extraction, followed by ethanol precipitation.

2.4.13 Estimation of DNA Concentration using Spectroscopy

DNA concentration of solutions was estimated using UV spectroscopy, as described by Sambrook et al. (1989). DNA was dissolved in 1 mL of $T_{10}E_1$ in a quartz crystal curvette (1 cm light path), and the UV absorbancy at 260 and 280 nm was measured using a diode array spectrophotometer (Hewlett Packard 8452A). When the ratio of absorbancies at OD_{260}/OD_{280} was equal to 1.8 ± 0.1, the concentration of dsDNA was calculated to be 50 ug mL^{-1} for every absorbance unit at OD_{260}.

2.5 AMPLIFICATION OF DNA BY PCR

2.5.1 PCR Primer Selection

DNA primers were selected for amplification of regions of yeast chromosomal DNA. Selection of appropriate primers was based on the following criteria. Primers had approximately 50% GC content. To prevent self-annealing, primer pairs were not complementary at the 3' ends. Primers did not have 3'-T as these have greater tolerance of a mismatch. Each primer had at least one A or T within the 3'-most triplet to prevent mismatch tolerance of primers with consecutive C's or G's. Gibco BRL Custom DNA primers were acquired from Life Technologies Ltd. The lyophilised primers were resuspended in 1 mL of $T_{10}E_1$ and stored at –20°C. Primers are listed in Appendix 3.

2.5.2 Amplification of DNA

Target regions of the yeast chromosome were amplified using the Expand™ High Fidelity Polymerase Chain Reaction (PCR) system (Boehringer Mannheim). PCR was carried out in thin-walled PCR tubes, in a total volume of 50 µL. This included 5 µL of Expand™ HF buffer (10x concentrated, with 15 mM $MgCl_2$), 5 µL of $MgCl_2$ Stock Solution (25 mM $MgCl_2$), 0.5 µL of Expand™ HF DNA Polymerase, 1 µL of PCR Nucleotide Mix (10 mM deoxynucleotide solution; Boehringer Mannheim), 100-200 pg each of 2 primers, and yeast chromosomal DNA. Amplification of DNA was performed in a PTC-150 MiniCycler™ with Hot Bonnet™ programmable thermal controller (MJ Research). The amplification program typically involved an initial incubation at 94°C for 5 min to denature DNA. This was followed by 33 cycles of denaturation at 94°C for 30 sec, primer annealing at 58°C for 30 sec, and primer extension at 72°C for 1 min. For reactions where the predicted PCR product was larger than 1.5 kb, the primer extension was carried out for an extra min per additional kb of predicted PCR product. The final cycle of amplification involved incubation at 94°C for 30 sec and 58°C for 30 sec as previously, finishing with a 5 min primer extension step at 72°C. Following PCR, amplified products were checked using agarose gel electrophoresis, and purified as necessary.

2.6 DNA SEQUENCE ANALYSIS

DsDNA sequence was determined using the LI-COR Automated DNA Sequencer, Model 4000L, involving infrared dye (IRD40) technology, or by using the ABI Prism Model 377 version 3.0 Automated Sequencer, employing Rhodamine terminator chemistry.

2.6.1 ABI Prism Automated Sequencing

When sequence information was required for PCR products, sequencing was performed using the ABI Prism Automated Sequencer at the Waikato DNA Sequence Facility (Department of Biological Sciences, The University of Waikato). PCR product template DNA and primer were supplied at concentrations of 0.2 μg μL^{-1} and 0.8 pM μL^{-1} respectively, in dH_2O.

2.6.2 LI-COR Automated DNA Sequencing

When DNA to be sequenced was cloned into the pBluescript® SK(+/-) multicloning site, sequencing was performed using the LI-COR Automated DNA Sequencer.

2.6.2.1 Cycle Sequencing Reaction

Cycle sequencing of template DNA was carried out using the IR Taq DNA Sequencing Kit for LI-COR Automated DNA Sequencers (Boehringer Mannheim).

Reaction preparations were carried out on ice, and kept in the dark as much as possible, by covering with foil. The following components were placed into a sterile tube: 1 pM of template DNA, 2 pM of M13/PUC 19mer IR sequencing forward primer (Appendix 3), 2 μL of reaction buffer, and dH_2O to a final volume of 19 μL. Following the addition of 1 μL of Taq polymerase (3 units), the reaction components were mixed. Volumes of 2 μL of each of the 4 long range sequencing termination mixtures were dispensed into 4 correspondingly labelled thin-walled PCR tubes. Aliquots of 4 μL of reaction components were added to, and mixed with, each of these termination mixes. Tubes were overlayed with 10 μL of mineral oil, and heated at 95°C for 5 min in a

MiniCycler™ with Hot Bonnet™ programmable thermal controller. Reaction tubes were then cycled through 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension/termination at 70°C for 1 min. The reaction was stopped by the addition of 2 μL of formamide buffer.

2.6.2.2 Sequencing Gel Electrophoresis

The LI-COR gel casting apparatus was assembled according to manufacturer's instructions, using LI-COR 66 cm glass plates (thoroughly cleaned). A 4% Long Ranger Gel solution was prepared, cast in the apparatus, and a 32-well rectangular tooth comb (4.5 mm) was added. Following 2 hr of gel polymerisation, the gel electrophoresis apparatus was assembled and the gel was pre-run for 1 hr in 1 x TBE* running buffer, using Version 2.31 Data Collection DEV7 software, and scanner control parameters listed in Appendix 2. Samples were denatured for 3 min at 95°C, and loaded into prewashed wells. Electrophoresis was carried out for 16 h. Results were visualised used the Version 2.30 Image Analysis program.

2.6.3 DNA Sequence Similarity Searches

DNA sequence data obtained was compared to sequences stored at the National Centre for Biotechnology Information, using FASTA format or blastn and blastx algorithms (Altschul et al., 1997; website http://www.ncbi.nlm.nihgov).

2.7 HYBRIDISATION OF RADIOLABELLED PROBES TO IMMOBILISED DNA

2.7.1 DNA Transfer to Membrane

DNA was immobilised from 0.7-1.0% agarose gels to Immobilon™-N membranes using a Hoefer TransVac™ Vacuum Transfer Unit.

Membranes were prewetted with 70% ethanol and rinsed with dH_2O prior to use. The transfer stack was assembled according to manufacturer's instructions and a vacuum of

5 cm Hg was applied and maintained throughout the procedure. The gel was covered with Depurination Solution and incubated for 10 min. Depurination Solution was replaced with Denaturation Solution, and incubated for 10 min. Denaturation Solution was removed and the gel was covered with Neutralisation Solution. After 10 min, this was removed and the gel was submerged with Transfer Solution (20 x SSC) for 90 min.

When transfer was complete, the membrane was dried on Whatman® 3MM paper, and DNA was cross-linked to the membrane using an Ultra-Lum UVC-515 ultraviolet multilinker, delivering an exposure of 0.12 J cm^{-2} at 254 nm.

2.7.2 Preparation of Labelled DNA Probe

Linear DNA to be used as a hybridisation probe was labelled with [α-^{32}P] dCTP using the Ready-To-Go™ DNA Labelling Kit (-dCTP) from Pharmacia Biotech.

Contents in the Reaction Mix tube were reconstituted in 20 µL of dH_2O and left on ice for 5 to 60 min. Linear DNA (25-50 ng in 25 µL dH_2O) was denatured by heating at 95-100°C for 3 min, and placed immediately on ice for 2 min. The denatured DNA was added to the reconstituted Reaction Mix, followed by 1-3 µCi of [α-^{32}P] dCTP (1-3 µL of 3000 Ci/mM). dH_2O was added to a final volume of 50 µL. The tube contents were mixed gently and were incubated at 37°C for 15 min.

2.7.3 Hybridisation of Labelled Probe to Immobilised DNA

The membrane containing immobilised DNA was prewetted in 95% ethanol and immersed briefly in dH_2O. After being rolled in mesh, it was placed into a Hybaid™ hybridisation tube, along with 10 mL of Prehybridisation buffer, which had been preheated to 68°C. The tube was incubated at 68°C, with constant rotation, in a Hybaid™ Micro-4 hybridisation oven. After at least 1 hr, the Prehybridisation solution was replaced with 10 mL of preheated Hybridisation solution, containing labelled hybridisation probe. Hybridisation was carried out for at least 16 hr, at 68°C.

Following hybridisation, unbound probe was removed by 3 washes. The first wash solution consisted of 100 mL of 2 x SSC and 0.1% SDS, and the two final washes contained 100 mL of 0.2 x SSC and 0.1% SDS. All wash solutions were preheated and each wash step was carried out for 15 min at 68°C. The washed membrane was dried on Whatman® 3MM paper, wrapped in plastic wrap, and exposed to a Kodak Storage Phosphor screen for 4 to 24 hr.

2.7.4 Detection

Kodak Storage Phosphor screens which had been exposed to membranes were scanned with the Molecular Dynamics Storm™ 840 system, Scanner Control version 4.00, build 54, using the Phosphor screen scanner type, at 740 V. Data was displayed and manipulated using the Molecular Dynamics ImageQuaNT™ Version 4.2a, Build 13 software package.

2.7.5 Removal of Bound Probe

Bound probe was stripped from membranes to be reprobed by incubation in 0.1 N NaOH for 30 min at RT. The membrane was then neutralised in STS for a minimum of 45 min at RT.

2.8 YEAST-YEAST MATING

Haploid yeast strains of opposite mating types were mated to create diploid variants as described by Sprague (1991).

A 1 mL volume from a saturated YPD culture of one of the strains to be mated was pelleted by centrifugation. Cells were resuspended in 1 mL of sterile dH_2O, centrifuged, and washed a second time in an analogous manner. After a third centrifugation, cells were resuspended in 1 mL of dH_2O, 100 µL of which was spread on media that selected for the growth of the diploid strain to be created only. The second strain in the mating pair, which had been grown on a YPD plate overnight, was

transferred to the mating plate using the replica plating method. Diploid variants arising after two to three days of growth at 30°C were restreaked to selective media for purification.

2.9 SPORULATION AND SPORE SEPARATION TECHNIQUES

2.9.1 Sporulation of Diploid Yeast

Diploid yeast strains to be sporulated were grown to saturation in YPD. The 10 mL culture was harvested by centrifugation and resuspended in 10 mL of sterile dH_2O. After a second centrifugation, cells were resuspended in 10 mL of McLary's medium (Spencer and Spencer, 1988) and incubated at 20°C in a 50 mL flask to provide adequate aeration. Ascus formation was observed by microscopy of lactophenol cotton blue-stained cell suspensions, and usually took 2 to 3 days.

2.9.2 Separation of Spores from Vegetative Cells

Sporulated cultures contained a combination of vegetative cells and tetrads of spores. Random spore analysis necessitated the disruption of ascus walls to free ascospores, and separation of spores from vegetative cells. This was accomplished using modifications of a variety of procedures outlined by Spencer and Spencer (1988).

Sporulated cultures were harvested by centrifugation and cells were resuspended in 10 mL of Pretreatment Solution. Following incubation at 37°C for 15 min, cells were washed once in 10 mL of 1.2 M Sorbitol Solution and repelleted. Cells were resuspended in 5 mL of Protoplasting Solution, and yeast lytic enzyme was added to make a final concentration of 1 mg mL^{-1}. Incubation was carried out at 37°C for 1 hr or until protoplasting was complete, as determined by microscopy. A volume of 10 mL of dH_2O was added, and the cell suspension was lightly centrifuged at 4 000 *x g* for 1 min to burst protoplasts of vegetative cells. The cell pellet was washed with 1 mL of dH_2O, and following centrifugation, was resuspended in another 1 mL of dH_2O. To eliminate any remaining vegetative cells, the predominantly spore suspension was incubated at

55°C, for 10 min. After heat treatment, spores still grouped in tetrads were separated by light sonication using a Microson™ ultrasonic cell disruptor (Heat Systems, Ultrasonics Inc), set on 30-40% power, for 30 s. The spore suspension was diluted and plated onto YPD plates for germination and growth.

2.10 SATURATION MUTAGENESIS OF COMPLEMENTING CLONES

Saturation mutagenesis of complementing clones was performed in a similar manner to methods described by Kleckner (1991). The element selected for mutagenesis was the mini-Tn10 derivative 103, conferring kanamycin resistance (Km^R) and housed in the phage vehicle, λNK1316.

A high titre of approximately 1 x 10^{10-11} pfu mL^{-1} of phage λNK1316 was prepared as follows, using a strain permissive for replication of the phage λ vehicle, *E. coli* PB2480. An overlay containing 3 mL of LB Top agar with 0.1 mL of a saturated culture of PB2480 grown in LBMM, was prepared on an LBMM plate. Volumes of 10 μL of various dilutions of phage stock were applied to the plate. A discrete plaque that formed on the plate was picked off and inoculated into 10 mL of LBMM, along with a 0.1 mL aliquot from a saturated culture of PB2480, also grown in LBMM. The culture was shaken vigorously at 37°C for approximately 4 to 6 h, until the culture cleared. Following addition of a few drops of chloroform, the culture was shaken and allowed to stand for 10 min. Following 10 min of centrifugation at 3000 *x g*, the supernatant consisting of high titre phage lysate was recovered.

The plasmids to be mutated were introduced into *E. coli* MC4100, a *sup0* strain that is not permissive for phage λ replication. Transformants, grown to saturation in LBMM, were concentrated to 10^{10} cells mL^{-1} in fresh LB. Phage was added to concentrated bacteria at varying multiplicities of infection of 0.1 to 1. Adsorption was carried out for 15 min at room temperature, followed by 15 min at 37°C. Cells were washed in LB containing sodium citrate. Proceeding elaboration in LB and sodium citrate for 1 h, cells were concentrated 5-fold and plated on media that selected for plasmid and

transposon presence. Colonies appearing were washed off plates and the plasmid DNA was recovered by the Alkaline Lysis Procedure.

3. RESULTS

3.1 TOXIC AMINO ACID ANALOG SELECTION

A characteristic of cells containing the *lup1* allele is their propensity to accumulate hydrophobic amino acids, particularly leucine, methionine and phenylalanine, better than progenitor Lup$^-$ cells, in the presence of ammonium (Heinemann et al., 1994). This characteristic was exploited to develop a selection for strains with the *LUP1* or *LUP1/lup1* genotypes. We reasoned that Lup$^+$ haploids would be differentially sensitive to toxic amino acids in ammonium-rich media, allowing us to select rare heterozygous diploids created when a *LUP1* allele was introduced into a *lup1* background. Lup$^+$ variants would accumulate higher concentrations of toxic analogs and thus be killed at lower concentrations than is required to kill *LUP1* or *LUP1/lup1* cells.

A number of amino acid analogs, known to inhibit growth and reproduction in yeast, were assessed for their effectiveness as a selection for the Lup$^-$ phenotype, using PGAs and MIC determinations (Section 2.3). Analogs included the methionine analogs L-methionine sulfoximine (Jung, 1985) and L-ethionine (Richmond, 1962; Farber, 1963; Gits and Grenson, 1967; Grenson et al., 1970), arginine analog L-canavanine (Richmond, 1962; Grenson et al., 1966; 1970), glutamine analog L-azaserine (Gale et al., 1972), and the phenylalanine analog *m*-fluoro-D,L-phenylalanine (Richmond, 1962; Wheatley, 1978; Fowden et al., 1967) (Figure 3.1). The medium used for PGAs and MICs was SD + 0.5 HULA. This medium circumvented, by using as low a concentration as possible, competitive inhibition of analog accumulation and/or use by exogenous amino acids. Strains 329-6C (*LUP1*), JY117 (*lup1*) and JY127 (*LUP1/lup1*) were compared simultaneously in the event that the Lup$^+$ phenotype was semidominant.

3.1.1 Plate Gradient Assays

Effectiveness of amino acid analogs as Lup$^-$ selection agents was initially assayed by PGAs, as described in Section 2.3.1 (Table 3.1).

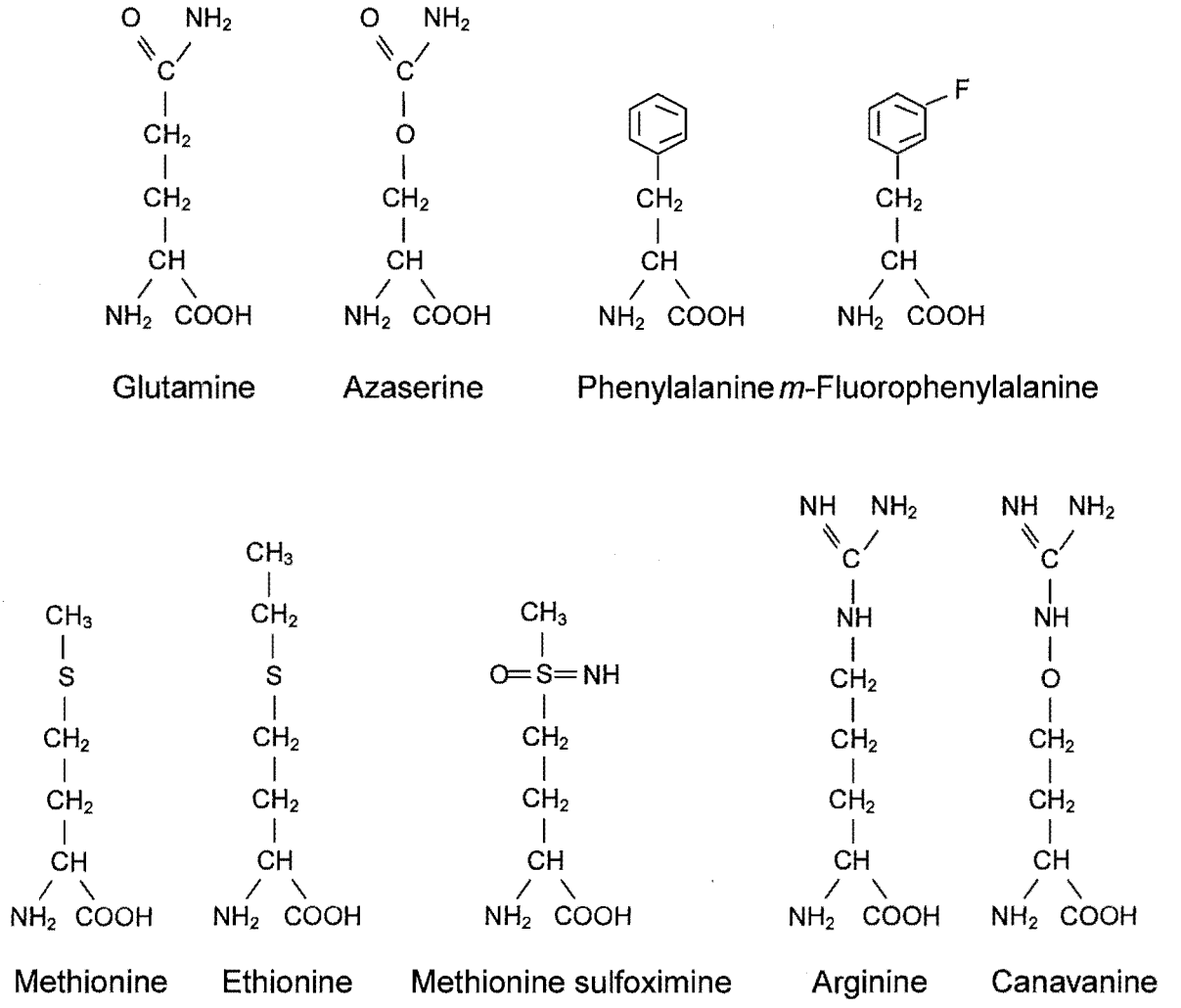

Figure 3.1: Comparison between amino acid and toxic analog structures.

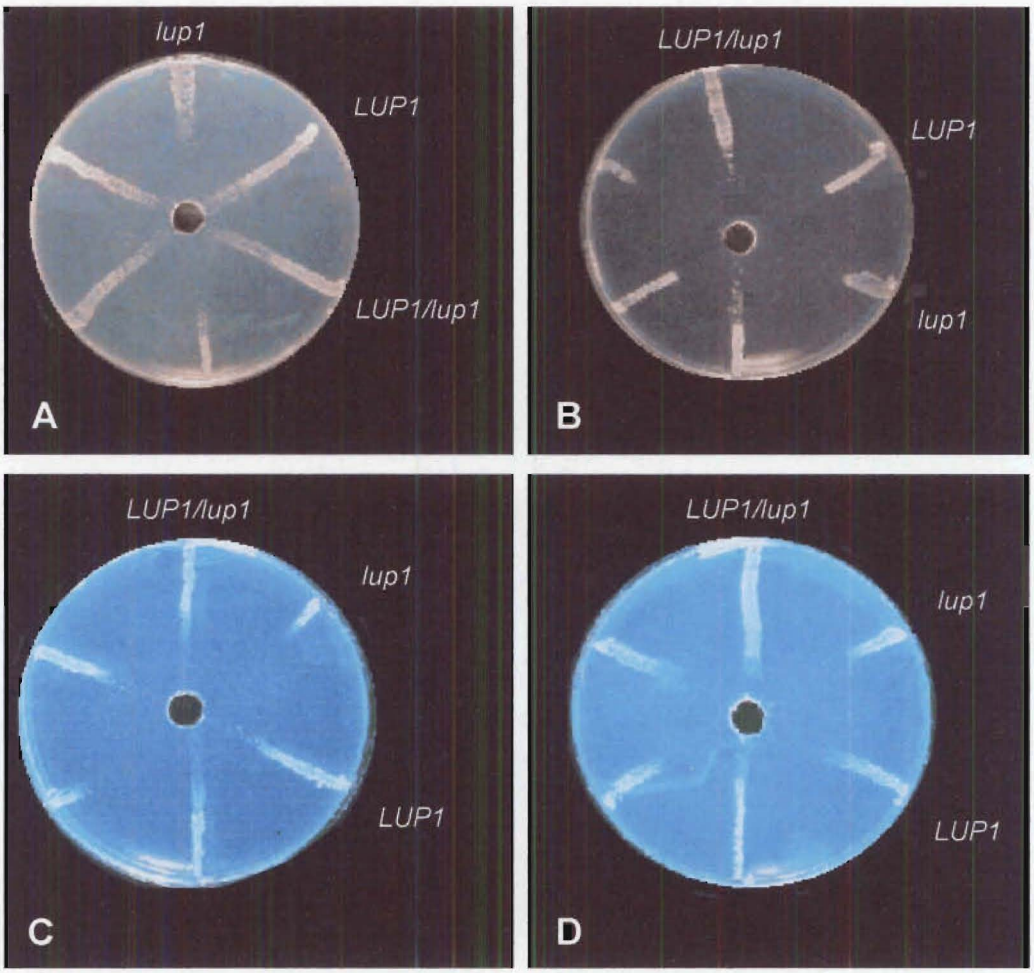

Figure 3.2: PGAs comparing sensitivities of JY117 (*lup1*), 329-6C (*LUP1*) and JY127 (*LUP1/lup1*) to toxic amino acid analogs. The toxic analog assayed in each plate include Plate A, L-azaserine; Plate B, L-canavanine; Plate C, *m*-fluoro-D,L-phenylalanine and Plate D, L-ethionine.

Lup$^-$ strains (JY127 and 329-6C) were more resistant than Lup$^+$ (JY117) to the amino acid analogs L-azaserine, L-ethionine, L-canavanine and *m*-fluoro-D,L-phenylalanine (Table 3.1, Figure 3.2).

3.1.2 Minimum Inhibitory Concentrations

The MICs of amino acid analogs for the growth of Lup$^+$, Lup$^-$ and *LUP1/lup1* strains were determined as outlined in Section 2.3.2 (Table 3.2).

MIC determinations were consistent with the PGA results. The JY117 MIC was lower than its Lup$^-$ counterparts for L-azaserine, *m*-fluoro-D,L-phenylalanine, L-canavanine, L-ethionine and L-methionine sulfoximine. The *LUP1/lup1* diploid, JY127, was resistant to similar or higher levels of all analogs than 329-6C, thus the Lup$^+$ phenotype does not appear to be semidominant.

Caution must be taken when interpreting results, however, as many genes may mediate levels of analog resistance of a strain, thus strains from different backgrounds may have different intrinsic resistance. JY127 is not isogenic with 329-6C, which could account for the anomalously high MIC for L-methionine sulfoximine. JY127 was created from a cross between a *lup1* strain, that was otherwise isogenic to 329-6C, and SY1229.

M-fluoro-D,L-phenylalanine was approximately 10 times more effective against Lup$^+$ than Lup$^-$ strains so SD + 0.5 HULA medium containing 15 mg L^{-1} of *m*-fluoro-D,L-phenylalanine (FPAM) was chosen to select for *LUP1* or *LUP1/lup1* types amongst *lup1* types, in subsequent experiments. The phenotype describing the ability of a strain to grow on this medium was designated Fpa^R, and sensitive strains were labelled Fpa^S.

Table 3.1: Results of PGAs comparing sensitivity of JY117, 329-6C and JY127 to toxic amino acid analogs. Results are expressed as averages of at least three independent ratios of distances in mm of growth inhibition from the plate centre after three days of incubation at 30°C on SD + 0.5 HULA medium.

Analog (Amount added per plate)	**Average Ratio of Zones of Inhibition From Plate Centre**	
	JY117:329-6C (*lup1*:*LUP1*)	**JY117:JY127** (*lup1*:*LUP1*/*lup1*)
L-azaserine (7.5 µg)	2.05 ± 0.38	1.42 ± 0.13
m-fluoro-D,L-phenylalanine (75 µg)	1.91 ± 0.55	1.93 ± 0.30
L-canavanine (7.5 µg)	1.48 ± 0.14	1.76 ± 0.26
L-ethionine (7.5 µg)	1.12 ± 0.16	1.52 ± 0.37

Table 3.2: MICs of toxic amino acid analogs. Medium for MICs comprised SD + 0.5 HULA.

Analog	**MIC (mg/L)**			**MIC Ratios**	
	JY117 (*lup1*)	**329-6C** (*LUP1*)	**JY127** (*LUP1*/*lup1*)	**JY117: 329-6C**	**JY127: 329-6C**
L-azaserine	0.05-0.06	0.2-0.25	0.25-0.3	0.2-0.3	1-1.5
m-fluoro-D,L-phenylalanine	2.5-5	20-25	20-30	0.1-0.25	1-1.5
L-canavanine	0.05-0.075	0.2-0.5	0.2-0.5	0.1-0.38	0.4-2.5
L-ethionine	0.075-0.1	0.3-0.4	1	0.19-0.33	2.5-3.3
L-methionine sulfoximine	0.025-0.05	0.4-0.6	30-40	0.04-0.13	50-100

3.2 CLONING OF *LUP1*

3.2.1 Isolating the *LUP1* Gene by Complementation

The initial step in cloning *LUP1* involved complementation of the Fpa^S phenotype of the recessive, *lup1*, allele using wildtype yeast genomic libraries. Two different yeast genomic libraries were obtained to minimise the possibility that they were made from Lup^+ strains, or had an underrepresentation of *LUP1*. The libraries were made in vectors with different copy numbers. The Rose library (Rose et al., 1987) is based on the low-copy number YCp50 plasmid (one to two copies per cell), generally the preferred vector when isolating a gene by complementation (Rose and Broach, 1991). The Botstein library (Carlson and Botstein, 1982) is based on the high-copy number vector, YEp24 (25-200 copies per cell).

Plasmid DNA from each of the different libraries was introduced into JY117, and transformants were selected on SC-URA medium, because the plasmid provided *URA3*. Using sterile toothpicks, transformants were transferred to YPD plates. JY117 and 329-6C colonies were included on plates as negative and positive controls, respectively. Following overnight growth, plates were replica-plated (Lederberg and Lederberg, 1952) to FPAM plates. Transformants found to be Fpa^R were purified by streaking onto FPAM plates. Plasmid DNA was extracted from purified transformants and introduced into *E. coli* DH10B for amplification. The mutation rate to the Lup^+ phenotype is purportedly high (Heinemann et al., 1994). To ensure that the Fpa^R phenotype of JY117 transformants was conferred by the library plasmid and not caused by a spontaneously occurring mutation within JY117, plasmid DNA was recovered from ampicillin-resistant (Ap^R) *E. coli* transformants, and used to transform naïve JY117. Ura^+ transformants were assayed as previously for the ability to grow on FPAM, as well as LLM.

A total of 9300 Ura^+ transformants derived from the Rose library were screened for the Fpa^R phenotype. Six transformants were found to be resistant. From a total of 7500 transformants containing plasmids from the Botstein library, 9 Fpa^R variants were isolated. Table 3.3 summarises the competence of specific plasmids isolated from Fpa^R transformants to retransform naïve JY117 to Fpa^R and to complement the Lup^+

phenotype. Plasmids recovered from all except one of the Fpa^R transformants conferred Fpa^R at a high frequency when inserted into naïve JY117 cells (100% of approximately 100 Ura^+ transformants were Fpa^R). All clones conferring Fpa^R from the Rose library and three from the Botstein library also rendered JY117 strains no longer Lup^+. JY117 remained Lup^+ when transformed with the remaining Fpa^R-conferring clones from the Botstein library, suggesting that more than one gene with the ability to confer Fpa^R had been isolated.

Table 3.3: Characteristics of library plasmids isolated from Fpa^R transformants.

Plasmid	Library Source	Ability to retransform JY117 to Fpa^R	Ability to complement Lup^+ phenotype of JY117
pJO13	Rose	Yes	Yes
pJO14	Rose	Yes	Yes
pJO15	Rose	Yes	Yes
pJO16	Rose	Yes	Yes
pJO17	Botstein	Yes	Yes
pJO18	Botstein	Yes	No
pJO20	Botstein	Yes	No
pJO21	Botstein	Yes	No
pJO22	Botstein	Yes	No
pJO23	Botstein	Yes	No
pJO24	Botstein	Yes	Yes
pJO25	Rose	Yes	Yes
pJO26	Rose	No	No
pJO27	Botstein	Yes	Yes
pJO28	Botstein	Yes	No

3.2.2 Plasmid Curing Studies

Further evidence that isolated plasmid clones confer Fpa^R comes from plasmid curing experiments. Yeast are easily cured of their plasmids by culture in media, like YPD, that is neutral to any gene on the plasmid (Singh and Heinemann, 1997). YPD cultures were inoculated with colonies from purified Fpa^R transformants, and grown to

saturation, diluted 1000-fold in fresh YPD and again cultured to saturation. Cultures were diluted and plated on YPD. Colonies arising were tooth-picked to YPD, along with the Fpa^S, Ura^+ control, JY117 containing YCp50, and Fpa^R, Ura^- control, 329-6C. Resulting colonies were replica-plated to SC-URA and FPAM plates.

All cells containing plasmids that, when isolated, could transform naïve JY117 to Fpa^R (Table 3.3), also had phenotypes that strictly correlated plasmid loss (Ura^- phenotype) with loss of the Fpa^R phenotype (Table 3.4; Figure 3.3). The results provided convincing evidence that in these cases, the origin of Fpa^R was plasmid-bourne. The strain containing pJO26, which was unable to transform naïve JY117 to Fpa^R, showed no correlation between plasmid presence and the Fpa^R phenotype, as all colonies remained Fpa^R irrespective of plasmid loss. In this strain, the Fpa^R phenotype was therefore likely mediated by spontaneous chromosomal mutation.

Table 3.4: Results of curing assays. Results show numbers of Ura^+, Fpa^R and Ura^+ and Fpa^R colonies following non-selective growth of JY117 transformed with the respective plasmids.

Plasmid	Ura^+	Fpa^R	Ura^+ and Fpa^R	Total Tested
pJO13	202	202	202	204
pJO14	183	183	183	189
pJO15	182	182	182	185
pJO16	90	90	90	95
pJO17	42	42	42	94
pJO18	104	104	104	175
pJO20	93	93	93	129
pJO21	105	104	104	130
pJO22	22	22	22	41
pJO23	103	103	103	168
pJO24	167	167	167	185
pJO25	171	171	171	175
pJO26	145	196	145	196
pJO27	90	90	90	195
pJO28	118	118	118	187

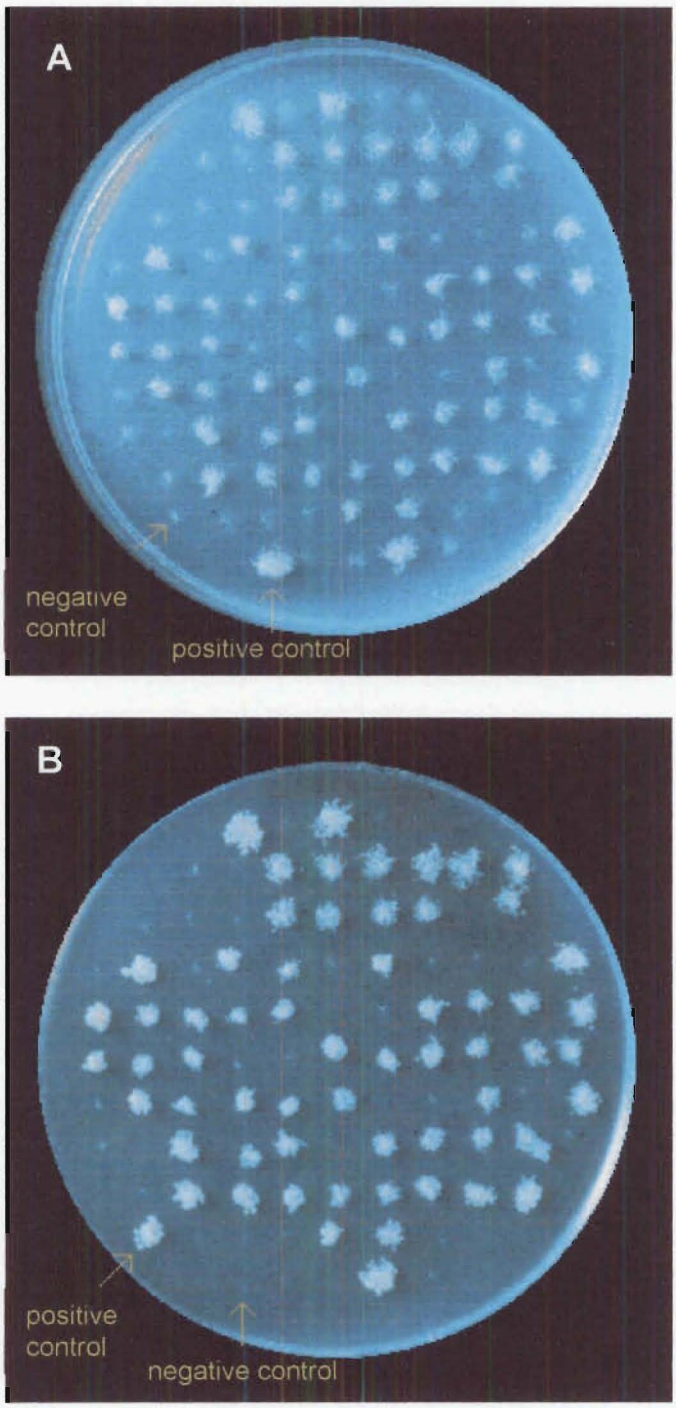

Figure 3.3: Curing assay showing total correlation between Fpa^R phenotype (Plate A) and maintenance of library plasmid, pJO28 (Ura^+ phenotype, Plate B). Controls included Fpa^S, Ura^+ JY117 transformed with YCp50, and Fpa^R, Ura^- 329-6C.

Table 3.5: Groups of identical complementing plasmids, determined from restriction enzyme analyses.

Representative Plasmid	Other Identical Plasmids
pJO13	pJO14, pJO15, pJO16, pJO25
pJO21	pJO18, pJO20, pJO22, pJO23, pJO28
pJO24	pJO27
pJO17	-

3.2.3 Restriction Mapping and Southern Analysis of Complementing Clones

The complementing clones comprised four groups (Table 3.5) based on restriction patterns generated from single digestions by the restriction enzymes *Eco*RV and *Hin*dIII.

More detailed restriction maps were constructed of the representative plasmids from each group, pJO13, pJO21, pJO17 and pJO24. Restriction sites were located by determining, via agarose gel electrophoresis, the lengths of fragments produced by single or double digests with a variety of restriction enzymes. DNA from the gels was transferred onto membranes, and Southern hybridisation analyses, using several restriction enzyme fragments from pJO13 and pJO21 as probes, augmented positioning of restriction sites on maps (for example, Figure 3.4).

The restriction maps of the complementing plasmids, not including vector DNA, are shown in Figure 3.5 (pJO13, pJO17 and pJO24) and Figure 3.6 (pJO21). Insert sizes were 11.6 kb (pJO13), 8.3 kb (pJO17), 7.6 kb (pJO24) and 7.5 kb (pJO21). Three plasmids, pJO13, pJO17 and pJO24, shared a common 7.4 kb region. Interestingly, both restriction and Southern hybridisation analyses suggested that pJO21 did not share any sequences with the other three plasmids, providing additional evidence that two separate genes complemented the Fpa^S phenotype of *lup1* strains. The three groups of plasmids sharing a common region were isolated from both yeast genomic libraries. However, the group of plasmids represented by pJO21 all originated from the Botstein library, and consisted of all clones that, whilst conferring Fpa^R to Lup^+ strains, did not

render these strains Lup$^-$. The *LUP1* coding region was therefore predicted to occur within pJO13, pJO17 and pJO24.

3.2.4 Subcloning

Complementation was used to identify the smallest subclone with Fpa^R activity (Figure 3.5). pJO31 and pJO32 were created by ligating the pJO13-bourne 2.3 and 3.6 kb *Bam*HI/*Eco*RI fragments, respectively, into YCp50, linearised by *Eco*RI and *Bam*HI. pJO48 consisted of religated pJO24 following a deletion of the 2.6 kb *Sac*I fragment. All insertions interrupted the tetracycline resistance gene of YCp50, thereby providing a means of screening for insertion events. Subclone constructions were confirmed by restriction enzyme analyses.

The only subclone produced that still complemented the Fpa^S phenotype of *lup1* mutants was pJO48 (Figure 3.5).

3.2.5 Saturation Mutagenesis of Complementing Clones

The ORFs of the complementing genes were further localised by saturation mutagenesis of pJO21 and pJO24 using the mini-Tn10 derivative 103, as described in Section 2.10.

Plasmid DNA, prepared from transposon-mutated pools of pJO21- or pJO24-containing bacteria, was used to transform competent *E. coli* MC4100 to kanamycin resistance (Km^R) and Ap^R. Plasmids isolated from resulting transformants were introduced into JY117, and transformants were assayed for maintenance or loss of Fpa^R. Transposon insertions were mapped by restriction analysis.

From 23 transposon-mutated derivatives of pJO24 isolated, two plasmids could no longer confer the Fpa^R phenotype (pJO33 and pJO34). Transposons inserted within approximately 500 bp of each other, and within a 1.1 kb *Eco*RV fragment (Figure 3.5). Similarly, 2 of 55 transposon mutants of pJO21 were isolated that did not complement the Fpa^S phenotype (pJO38 and pJO39). Transposons inserted within a distance of approximately 500 bp, in the 2.2 kb *Eco*RV/*Pvu*II fragment of pJO21 (Figure 3.6).

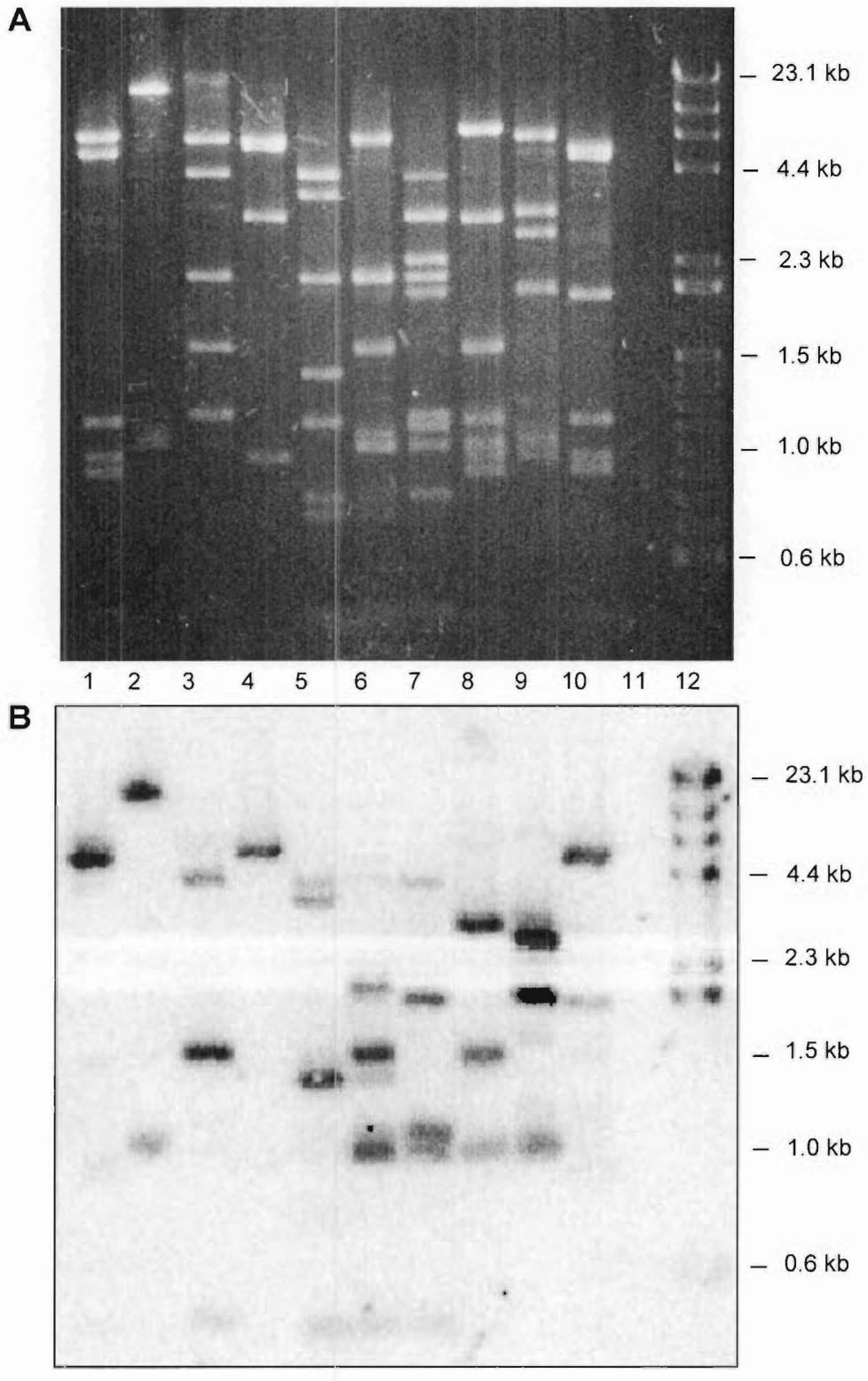

Figure 3.4: Restriction enzyme (A) and Southern (B) analysis of pJO24. DNA in lanes was digested with the following enzymes: lane 1, *Eco*RV; lane 2, *Bgl*II, lane 3, *Hin*dIII; lane 4, *Pvu*II; lane 5, *Hin*dIII/*Eco*RV; lane 6, *Hin*dIII/*Bgl*II; lane 7, *Hin*dIII/*Pvu*II; lane 8, *Bgl*II/*Eco*RV; lane 9, *Bgl*II/*Pvu*II; lane 10, *Eco*RV/*Pvu*II; lane 12, 100 bp ladder (Gibco BRL) and λ digested with *Hin*dIII. The autoradiogram in (B) resulted from probing DNA from the above gel (A) with [α-^{32}P]dCTP-labelled λ *Hin*dIII and 5.7 kb *Pvu*II fragment from pJO13.

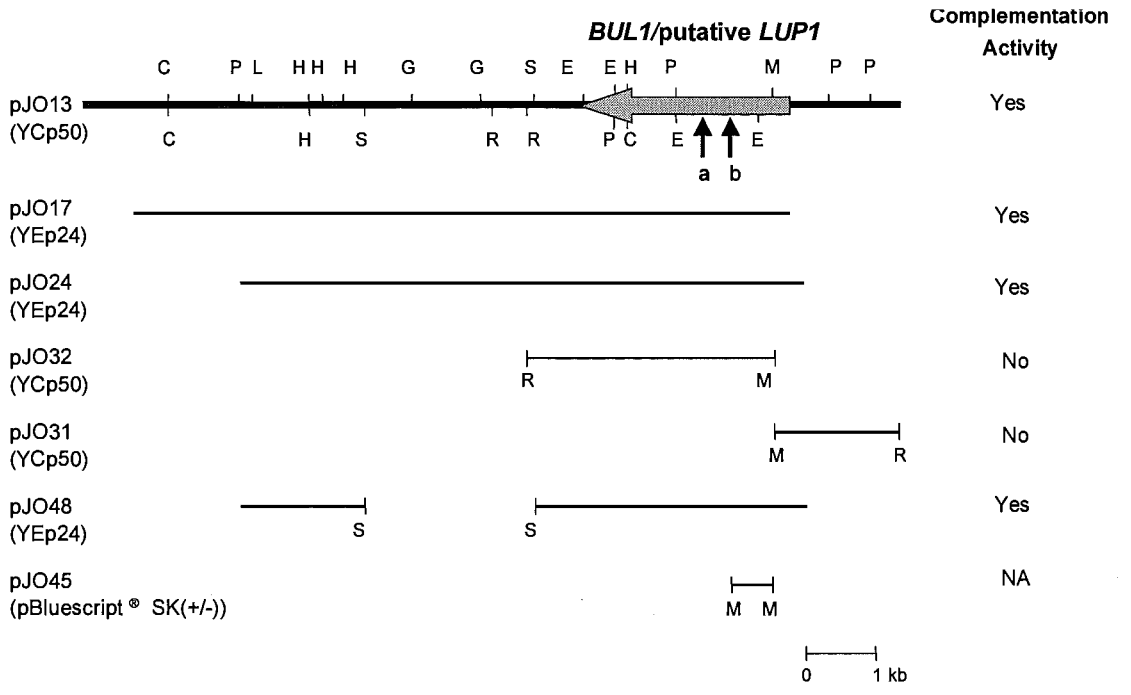

Figure 3.5: Restriction map of *BUL1*/putative *LUP1* and neighbouring regions. Lines represent DNA inserted within various vectors (in brackets). Production or source of various plasmids is detailed in the text. Ability to complement the Fpa^S phenotype is indicated. pJO45 was constructed for sequencing purposes using pBluescript® SK(+/-), a vector that is unable to replicate in yeast, thus complementation activity of this plasmid was not applicable (NA). Vertical arrows within pJO13 represent positions where transposon insertion eliminated Fpa^S and Lup^+ complementing ability. Transposons inserted at position (a) within pJO33, and at (b) in pJO34. The exact position of the *BUL1* coding region (denoted by the horizontal arrow) was determined from published sequence information of the *BUL1* region.
Abbreviations for restriction endonuclease sites include C, *Cla*I; L, *Sal*I; P, *Pvu*II; H, *Hin*dIII; G, *Bgl*II; R, *Eco*RI; E, *Eco*RV; M, *Bam*HI; S, *Sac*I. Exact fragment sizes are listed in Appendix 4.

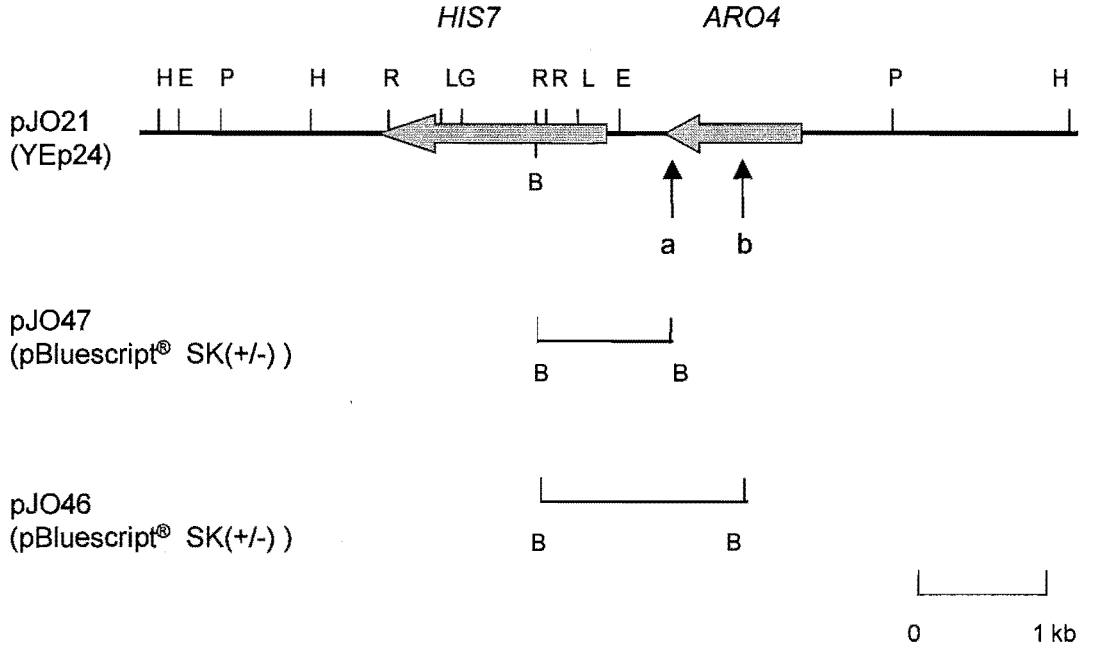

Figure 3.6: Restriction map of pJO21 showing *HIS7*, *ARO4* and neighbouring regions. Shown is DNA inserted within the *Bam*HI site of YEp24. Vertical arrows denote positions within which transposon insertion eliminates ability to complement the Fpa^S phenotype. Transposon insertion occurs at position (a) within pJO39 and position (b) within pJO38. Also shown is region of DNA cloned from pJO39 and pJO38 to create plasmids pJO47 and pJO46, respectively, used for sequencing. Details of plasmid construction are described in the text. Horizontal arrows denote position and direction of transcription of *HIS7* and *ARO4*, determined from published sequence information.
Abbreviations for restriction endonuclease sites include B, *Bam*HI; P, *Pvu*II; H, *Hin*dIII; G, *Bgl*II; R, *Eco*RI; E, *Eco*RV; L, *Sal*I.
Fragment sizes are listed in Appendix 4.

3.2.6 DNA Sequencing of Complementing Genes

A transposon terminus in pJO34 was selected as the initiation point for DNA sequencing of the putative *LUP1* coding region. A 0.5 kb *Bam*HI fragment, containing the transposon terminus and putative *LUP1* DNA, was isolated from pJO34 and inserted into the *Bam*HI restriction site in pBluescript® SK(+/-), to create pJO45 (Figure 3.5). Sequencing was carried on the LI-COR Automated DNA Sequencer out using the M13/pUC 19-mer forward primer (Appendix 3). 390 bases of the resulting sequence corresponded to non-transposon or vector DNA.

The DNA sequence of the predicted *LUP1* allele was identical to *BUL1* (GenBank database accession number D50083; Yashiroda et al., 1996), *DAG1* (GenBank accession number L40587; Biggins et al., 1996), *ZZZ1* (Wolfe et al., 1999) and *RDS1* (GenBank accession number X88901) (Figure 3.20). *BUL1* is a 3 kb gene, with a predicted gene product that appears to be a basic and hydrophilic 110 kDa protein.

As with the *LUP1* sequencing approach, *Bam*HI fragments from pJO38 and pJO39, containing one transposon end and its adjacent DNA, were cloned into the *Bam*HI site of pBluescript® SK(+/-). A 1.5 kb fragment was isolated from pJO38 to create pJO46, and a 1 kb fragment was recovered from pJO39 to create pJO47 (Figure 3.6). DNA was sequenced using the M13/pUC 19-mer forward primer, as for pJO45. Initial sequence information obtained from pJO47 contained the transposon insertion sequence, thus we predicted that the 89 adjacent bases obtained consisted of the part of gene responsible for complementation of Fpa^S. This DNA was highly similar to *ARO4* (GenBank database accession number X61107; Figure 3.7). The orientation of the *Bam*HI fragment in pJO46 was in the reverse direction with respect to the M13/pUC 19-mer region on pBluescript® SK (+/-). Sequence information obtained from pJO46, therefore, did not necessarily correspond to the gene responsible for complementation. In fact, the 660 bases obtained were identical to part of *HIS7* (GenBank database accession number Z36117; Figure 3.8), which is separated from *ARO4* by a 417 bp intergenic region (Springer et al., 1997). *ARO4* therefore comprises the gene present on pJO21 that is able to complement the Fpa^S, but not Lup^+, phenotype.

Figure 3.7: Comparison between sequence information obtained from pJO47, and *ARO4* (GenBank database accession number X61107). The letter n represents bases that were too ambiguous to be identified. Numbers beside *ARO4* sequence represent the number of bases from the beginning of the *ARO4* coding region.

```
pJO47: 1     tgcttgtatagggtgggaaactactgaagacggctngaggaaattggctgctgctgncag
             ||||||||||||| |||||||||||||||||||| || ||||||||||||||||||| |||
ARO4: +1026 tgcttgtataggttgggaaactactgaagacgtcttgaggaaattggctgctgctgtcag

pJO47: 61    acaaagaagagaagnnaacaagaaanaga
             ||||||||||||||  ||||||||| |||
ARO4: +1115 acaaagaagagaagttaacaagaaataga
```

Figure 3.8: Comparison between sequence information obtained from pJO46, and *HIS7* (GenBank database accession number Z36117). Numbers beside *HIS7* sequence represent the number of bases from the beginning of the *HIS7* coding region.

```
pJO46: 1     ggatccaatccaaagaataggttttccgagggaatgcaagaattccaacctatttctggt
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: +416   ggatccaatccaaagaataggttttccgagggaatgcaagaattccaacctatttctggt

pJO46: 61    actggcttttctgaatcatcgaacctggacaacttaaaatcaatgtagttcagacccgta
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: +356   actggcttttctgaatcatcgaacctggacaacttaaaatcaatgtagttcagacccgta

pJO46: 121   ctcttagggctttccacggaaccggcaaagagcgcttgtagcccgacgcaaattcccatt
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: +296   ctcttagggctttccacggaaccggcaaagagcgcttgtagcccgacgcaaattcccatt

pJO46: 181   attggttttccagattcaatgtattctcttatcggcttttcgaatcctctattaaataaa
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: +236   attggttttccagattcaatgtattctcttatcggcttttcgaatcctctattaaataaa

pJO46: 241   ttgtcgacgaaatggccataatttccgacaccaggcaaaatcaatcttgacgtgcctgat
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: +176   ttgtcgacgaaatggccataatttccgacaccaggcaaaatcaatcttgacgtgcctgat

pJO46: 301   atgttaaaatcctttggtgatttcaccagttgtacttcgtaacctaaatgctcaattgca
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: +116   atgttaaaatcctttggtgatttcaccagttgtacttcgtaacctaaatgctcaattgca

pJO46: 361   ttggttagtgactgtaggttaccactttcaacgtcaatcacgtgaacgaccggcattctc
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: +56    ttggttagtgactgtaggttaccactttcaacgtcaatcacgtgaacgaccggcattctc

pJO46: 421   tttttctttacttgtaaataattaaaaaaacctaaactggatactgctacttcaatagctg
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: -5     tttttctttacttgtaaataattaaaaaaacctaaactggatactgctacttcaatagctg

pJO46: 481   cctcttttctttaaaacctgattgagtagtcgtcgatatcaaaggaatatcaacttatg
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: -65    cctcttttctttaaaacctgattgagtagtcgtcgatatcaaaggaatatcaacttatg

pJO46: 541   tatgtttcgatgtctgactcttttctcatgaatttttcatttttcatgatcacctaatt
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: -125   tatgtttcgatgtctgactcttttctcatgaatttttcatttttcatgatcacctaatt

pJO46: 601   agccgtggagagagagaaaaataatcccaaagctacgatatgactcaatttttttttgca
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HIS7: -185   agccgtggagagagagaaaaataatcccaaagctacgatatgactcaatttttttttgca
```

3.2.7 Levels of Complementation of Fpa^S and Lup^+ Phenotypes by Complementing Plasmids

The levels at which pJO13, pJO24, pJO17, pJO21 and the neutral plasmid Ycp50 could complement the Lup^+ and/or Fpa^S phenotypes of JY117 (*lup1*) were determined to compare differences in complementing abilities of the *ARO4* and *BUL1* genes, and of *BUL1* when expressed on high and low copy number vectors.

Results of MICs of *m*-fluoro-D,L-phenylalanine in SD + 0.5 HUTAL media for growth of JY117 containing the various plasmids revealed that *ARO4*, when expressed on a high copy number vector (pJO21), was best able to complement the Fpa^S phenotype of JY117. The MIC for JY117 containing pJO21 was 70 mg mL^{-1}, compared with 10-15 mg mL^{-1} for JY117 containing Ycp50 (Table 3.7). *BUL1* could better complement the Fpa^S phenotype of JY117 when expressed from a low copy number plasmid (pJO13) than from high copy number plasmids (pJO17 and pJO24), with MICs of 30, 25-30 and 20, respectively. It remains possible, however, that differences in expression may also be influenced by the different amounts of DNA flanking *BUL1* on each plasmid (see Figure 3.5).

As we have seen previously, *ARO4* was unable to complement the Lup^+ phenotype of JY117. The minimum concentration of leucine that JY117 could grow at was 2.5 mg mL^{-1}, when it carried either pJO21 or Ycp50 (Table 3.7). Like complementation of the Fpa^S phenotype, *BUL1* was best able to complement the Lup^+ phenotype of JY117 when expressed from low copy number pJO13. Minimum concentrations of leucine required for growth of JY117 containing pJO13, pJO17 and pJO24 were 10, 5 and 5 mg mL^{-1}, respectively.

The *m*-fluoro-D,L-phenylalanine MICs and minimum leucine concentrations for growth of JY117 containing pJO13 closely resemble profiles for 329-6C (Table 3.7). These results support our prediction that *LUP1* is allelic to *BUL1*.

3.3 *LUP1* IS ALLELIC TO *BUL1*

3.3.1 *BUL1* Knock-out Analysis

BUL1 complementation of the Lup^+ and Fpa^S phenotypes alone is insufficient evidence to conclude that *LUP1* and *BUL1* are allelic. It is also necessary to demonstrate that *bul1* mutants display the same phenotypes as *lup1* mutants. To this end, a knock-out mutant was created at the chromosomal locus homologous to cloned *BUL1*.

A disrupted *BUL1* locus in 329-6C was created as follows (Figure 3.9). A 1.45 kb *Cla*I/*Sac*I fragment from plasmid pJO13, containing part of the *BUL1* coding region, was introduced into *Sac*I/*Cla*I-digested pBluescript® SK(+/-), to create pJO58. pJO59 originated from elimination of a 0.43 kb *Eco*RV fragment from the *BUL1* coding region of pJO58. A 1.17 kb *Hin*dIII fragment from YEp24, containing *URA3*, was inserted into the *Hin*dIII site of pJO59 to create pJO60. pJO60 was digested with *Sac*I and *Cla*I and the liberated 2.19 kb fragment, containing the partially deleted *BUL1* gene flanking the *URA3* gene, was introduced into 329-6C. Ura^+ transformants resulted from recombination of the linear construct into the host chromosome. These were tested for the ability to grow on FPAM and LLM.

A majority of transformants assayed had acquired the Lup^+ and Fpa^S phenotypes, (Figure 3.10) although several remained Fpa^R and Lup^-.

To confirm that the knockout construct had partially replaced *BUL1* in Lup^+ integrants, the predicted region of integration and flanking areas were amplified by PCR, from chromosomal DNA of both Lup^+ (JOY64-67) and Lup^- (JOY62, JOY63) integrants, and control 329-6C. Primers for amplification included JO5 and JO9 (Appendix 3). PCR products, undigested and digested independently with *Sac*I/*Cla*I and *Eco*RV, were examined using agarose gel electrophoresis (Figure 3.11). Sizes of restriction enzyme-digested and undigested PCR products from 329-6C and Lup^- integrants were identical, indicating that the linear construct had not integrated within *BUL1* in Lup^- integrants. Sizes of fragments obtained from digestion of PCR products amplified from Lup^+ integrants mirrored the size of fragments that would be expected if the linear construct had recombined at its homologous ends, and replaced the region within *BUL1* to downstream of *BUL1*.

DNA from the gel was transferred onto a nylon membrane and probed with the 1.17 kb *Hin*dIII fragment containing *URA3* (from YEp24). The probe only hybridised to DNA fragments from Lup^+ integrants predicted to contain *URA3* assuming correct replacement by the linear construct had occurred (Figure 3.11). The regions amplified by PCR from 329-6C, Lup^- and Lup^+ integrants, as deduced by restriction enzyme and Southern analyses, are depicted in Figure 3.12.

The correlation between acquisition of the Lup^+ phenotype and replacement of the chromosomal *BUL1* allele with a partially deleted copy of *BUL1*, supports the notion that *BUL1* and *LUP1* are allelic.

3.3.1.1 Position of Integration in Lup^- Transformants

One tenth of the cells remained Lup^- following integration of the linear construct. In these transformants, the linear construct may have inserted downstream of *BUL1* at the region homologous to one of the termini of the construct, resulting in a partial duplication of the *BUL1* gene, instead of replacing *BUL1*. Restriction and Southern analyses revealed that this had not occurred, however (Figure 3.11).

DNA sequence of linear construct termini was compared to sequences stored at the National Centre for Biotechnology Information, using the blastn algorithm (Altschul et al., 1997). Interestingly, the termini with approximately 100 bases judged to be homologous to *BUL1*, was highly similar to sequence within *S. cerevisiae* chromosome XIII cosmid 8339 which corresponds to the *BUL2* gene (GenBank accession number Z49210). The first 79 nucleotides of the construct had 73% identity to *BUL2* sequence, and the sequence comparison is shown in Figure 3.13. It is therefore possible that integration had occurred within *BUL2* in Lup^- integrants.

To investigate whether the DNA had integrated at *BUL2*, *BUL2* and flanking regions were amplified from chromosomal DNA of 11 Lup^- integrants (JOY62, 63, 80-88), one Lup^+ integrant (JOY64) and 329-6C, using PCR. PCR primers included JO11 and JO12 (Appendix 3). The PCR product of the *BUL2* region would be larger than that of the wildtype if the linear construct has inserted within, or partially replaced, *BUL2*. The size of PCR products from all strains was found to be 4.5 kb, however. Therefore, integration had not occurred within *BUL2* in Lup^- transformants.

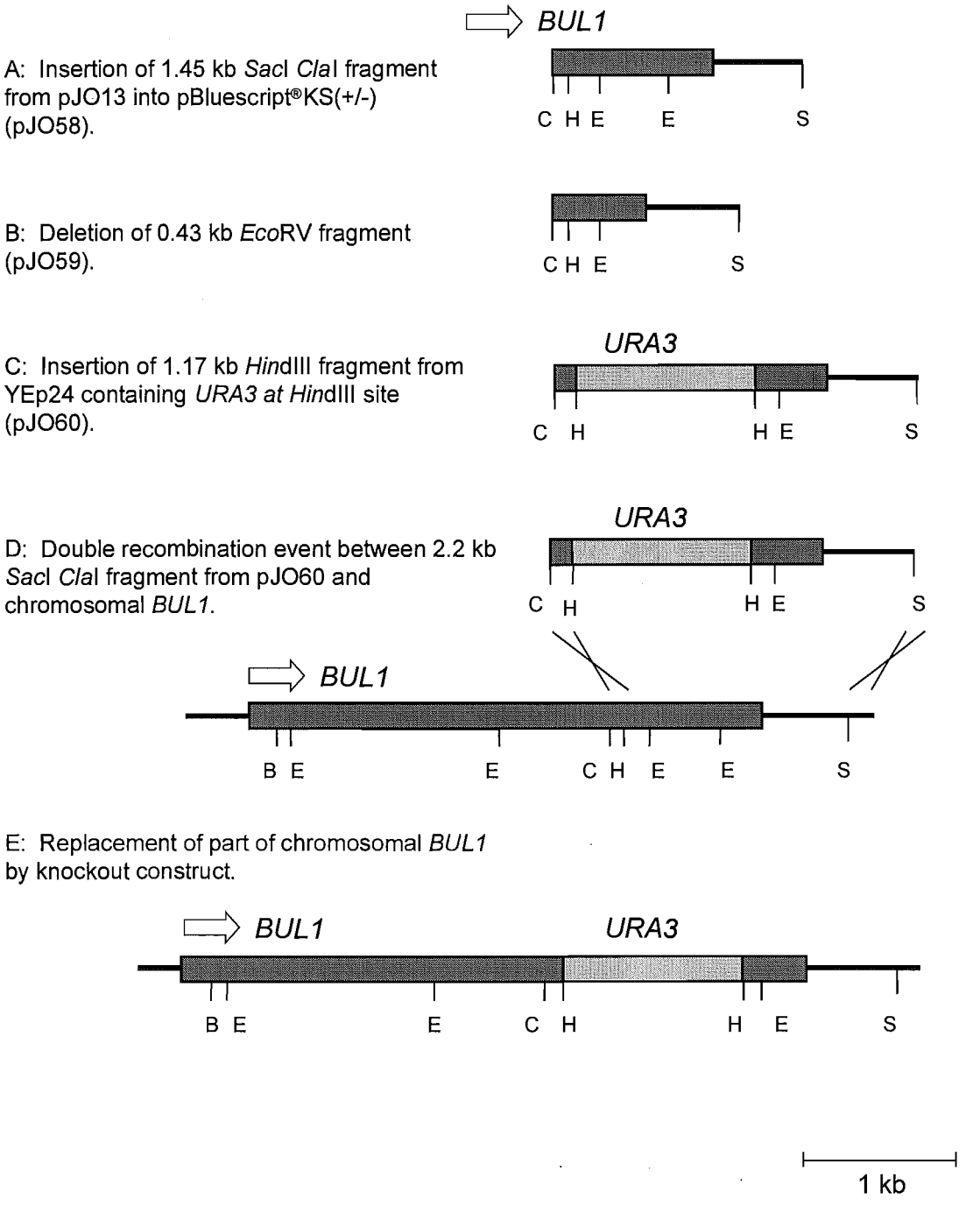

Figure 3.9: Construction of *LUP1* knockout construct consisting of a 0.43 kb *Eco*RV deletion from the 1.45 kb *Cla*I/*Sac*I fragment, and the insertion of a 1.17 kb fragment containing *URA3*. Abbreviations for restriction endonuclease sites: H, *Hin*dIII; R, *Eco*RI; E, *Eco*RV; B, *Bam*HI; S, *Sac*I; C, *Cla*I.

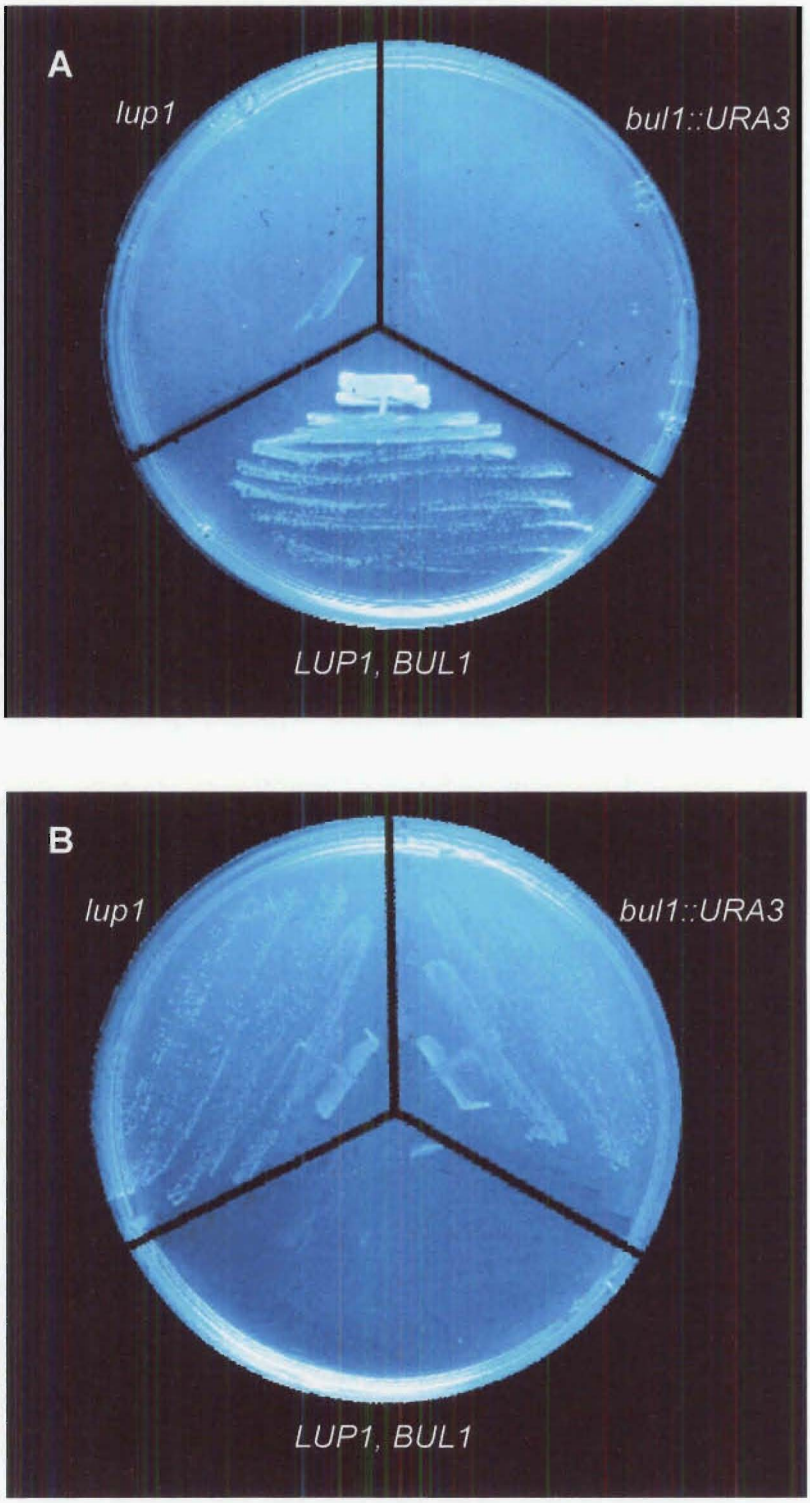

Figure 3.10: Demonstration of acquisition of Fpa^S (Plate A) and Lup$^+$ (Plate B) phenotypes by the partial deletion of the chromosomal *BUL1* allele. Media was FPAM for Plate A and LLM for Plate B. Strains included JY117 (*lup1*), 329-6C (*LUP1, BUL1*) and JOY64 (*bul1::URA3*).

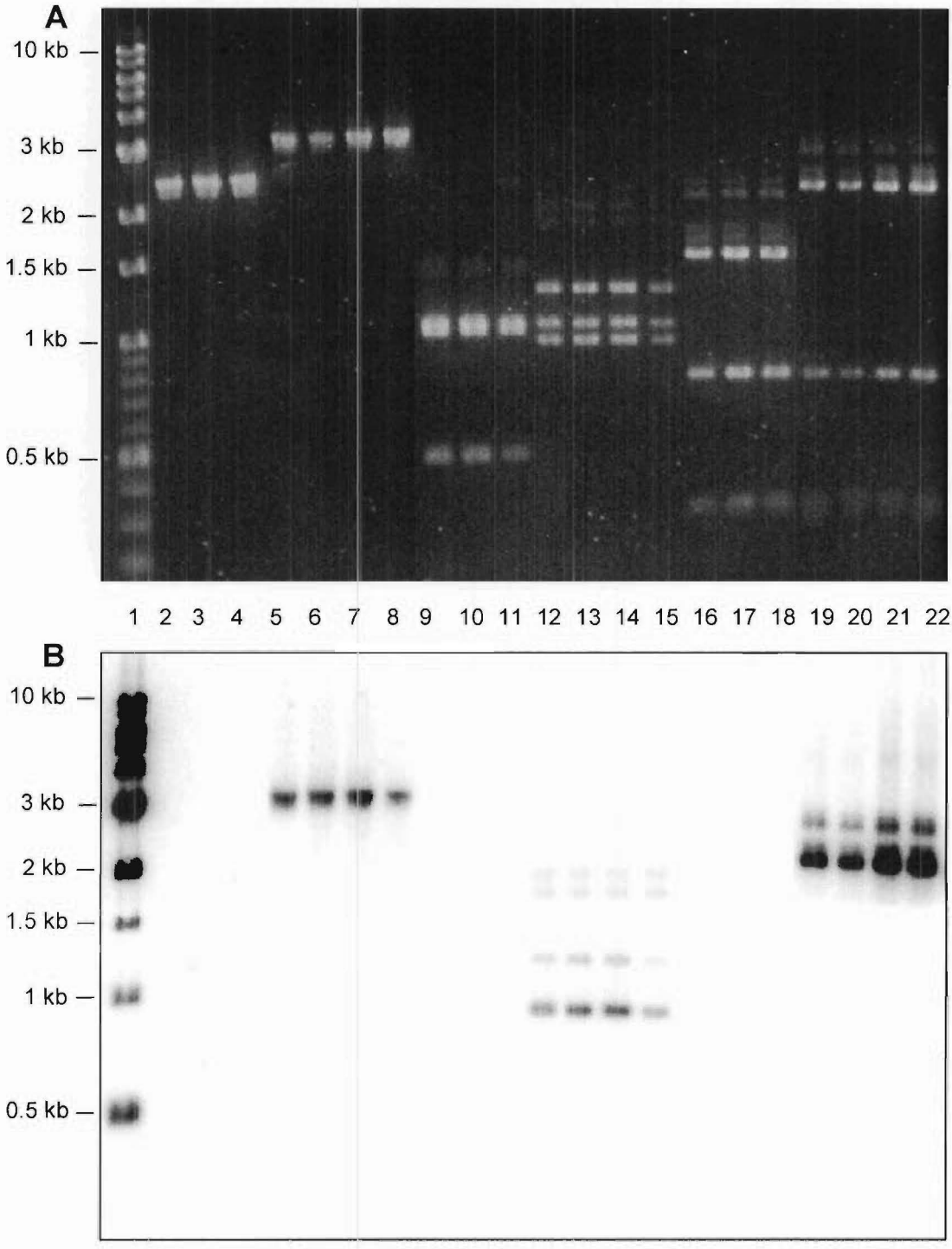

Figure 3.11: Restriction enzyme (A) and Southern (B) analyses of DNA amplified by PCR using primers JO5 and JO9, from strains that had integrated the knockout construct. DNA in lanes is from the following sources: lane 1 - 1 kb (New England Biolabs) and 100 kb ladder (Bio-rad); lanes 2, 9, 16 – 329-6C control; lanes 3, 10, 17 - JOY62 (Lup^+); lanes 4, 11, 18 - JOY63 (Lup^-); lanes 5, 12, 19 - JOY64 (Lup^+); Lanes 6, 13, 20 - JOY65 (Lup^+); lanes 7, 14, 21 - JOY66 (Lup^+); lanes 8, 15, 22 - JOY67 (Lup^+). Lanes 2 to 8 are undigested PCR products, Lanes 9 to 15 are PCR products digested with *Eco*RV and Lanes 16-22 are PCR products digested with *SacI*/*Cla*I. The probes for the Southern blot (B) consisted of the 1.17 kb *Hin*dIII *URA3* fragment from YEp24, and the 1 kb ladder, both of which were labelled with [α-^{32}P]dCTP.

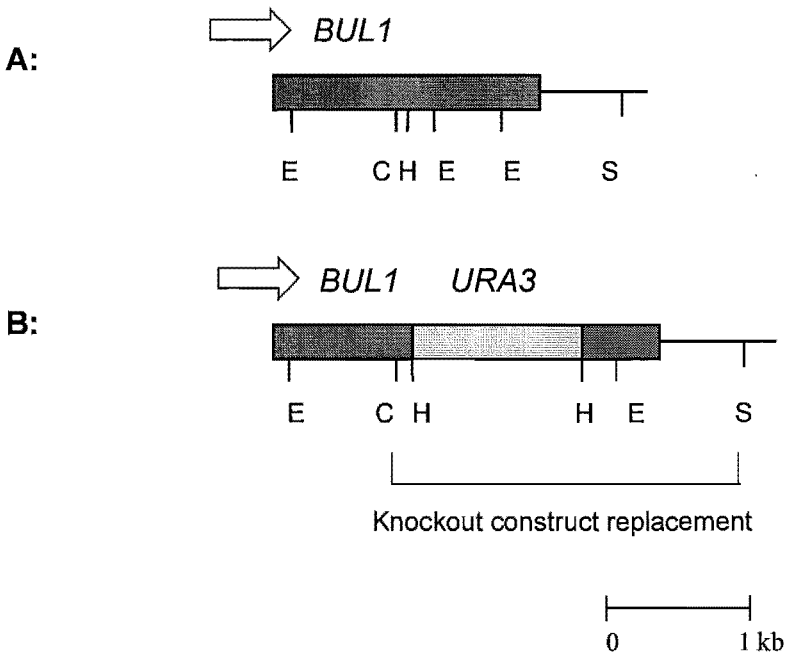

Figure 3.12: Diagram showing region amplified by PCR.
A: Region from 329-6C (wildtype) and Lup$^-$, Ura$^+$ integrants amplified by PCR, consisting of 1.45 kb of the 3' end of the *BUL1* coding region (shaded in dark grey) and 1 kb of DNA downstream of *BUL1*.
B: Region from Lup$^+$, Ura$^+$ integrants amplified by PCR. The area where partial replacement of chromosomal *BUL1* has occurred is shown.
Abbreviations for restriction endonuclease sites include H, *Hin*dIII; R, *Eco*RI; E, *Eco*RV; B, *Bam*HI; S, *Sac*I; C, *Cla*I.

```
BUL1:  +2207  TTACCACAGAACTGATATGTATAACAGCTAAATCTGATAATTCTATCCCAATAAAACTCA
               | || |||||| |  |    || ||||| || ||||||||||| || || || ||| ||
BUL2:  +2074  TAACGACAGAATTTGTGGTCATCACAGCAAAGTCTGATAATTCGATTCCTATCAAATTCT

BUL1:  +2267  ATTCTGAACTGTTGATGAA
                 ||||||||||||||||
BUL2:  +2134  GCACTGAACTGTTGATGAA
```

Figure 3.13: Comparison between *BUL2* coding sequence and *BUL1* coding sequence occurring at terminus of knockout construct. Numbers represent number of bases from beginning of *BUL1* or *BUL2* coding region.

3.3.2 Is *BUL2* Allelic to *LUP1*?

In addition to having 51% amino acid identity, *BUL1* and *BUL2* confer similar phenotypes. *BUL2*, when expressed on a single copy vector, could suppress temperature sensitivity of *bul1* disruptants. Whilst temperature-, high salt- and glycerol-sensitivity of *bul1* variants was strain-dependent, these phenotypes were detected regardless of strain background when *BUL1* and *BUL2* were simultaneously disrupted (Yashiroda et al., 1998). It is therefore possible that *BUL2* is allelic to *LUP1*.

If *BUL2* is allelic to *LUP1*, disruption of *BUL2* should render cells Lup^+ and Fpa^S. Furthermore, *BUL2* should complement the Lup^+ and Fpa^S phenotypes of *lup1* variants. PGAs and MICs using the inhibitor *m*-fluoro-D,L-phenylalanine were therefore compared for the *bul2* disruptant, YHY007K, progenitor strain, KA31-2A, and the *bul1 bul2* double disruptant, YHY008K. Sensitivity to *m*-fluoro-D,L-phenylalanine was also compared for JY117 transformed with pHY32, a low-copy number plasmid containing *BUL2*, as well as JY117 containing YCp50, and JY117 containing pJO13. The lowest concentration of leucine that could support colony formation after incubation for three days was also compared for each of the strains.

PGAs (Figure 3.14, Table 3.6) and MIC determinations (Table 3.7) revealed that YHY007K (*bul2*) was no more sensitive to *m*-fluoro-D,L-phenylalanine than was KA31-2A (*BUL1 BUL2*) (Figure 3.15). Both were sensitive to 60 mg L^{-1} of *m*-fluoro-D,L-phenylalanine when added to SD + 0.5 HULAT medium, whereas the otherwise isogenic, *bul1 bul2* mutant, YHY008K, was sensitive to 20-30 mg L^{-1} of this analog. Moreover, the concentration of leucine required for colony formation by KA31-2A and YHY007K was 7.5 mg L^{-1}, compared with 2.5 mg L^{-1} for YHY008K (*bul1 bul2*) and JY117 (*lup1*) (Table 3.7, Figure 3.15). Expression of *BUL2* from pHY32 was also incapable of complementing the Fpa^S and Lup^+ phenotypes of JY117. The lowest concentrations of *m*-fluoro-D,L-phenylalanine to which JY117 was sensitive, and lowest concentrations of leucine at which this strain could form colonies, were unchanged when pHY32 was present. Therefore *LUP1* does not appear to be allelic to *BUL2*.

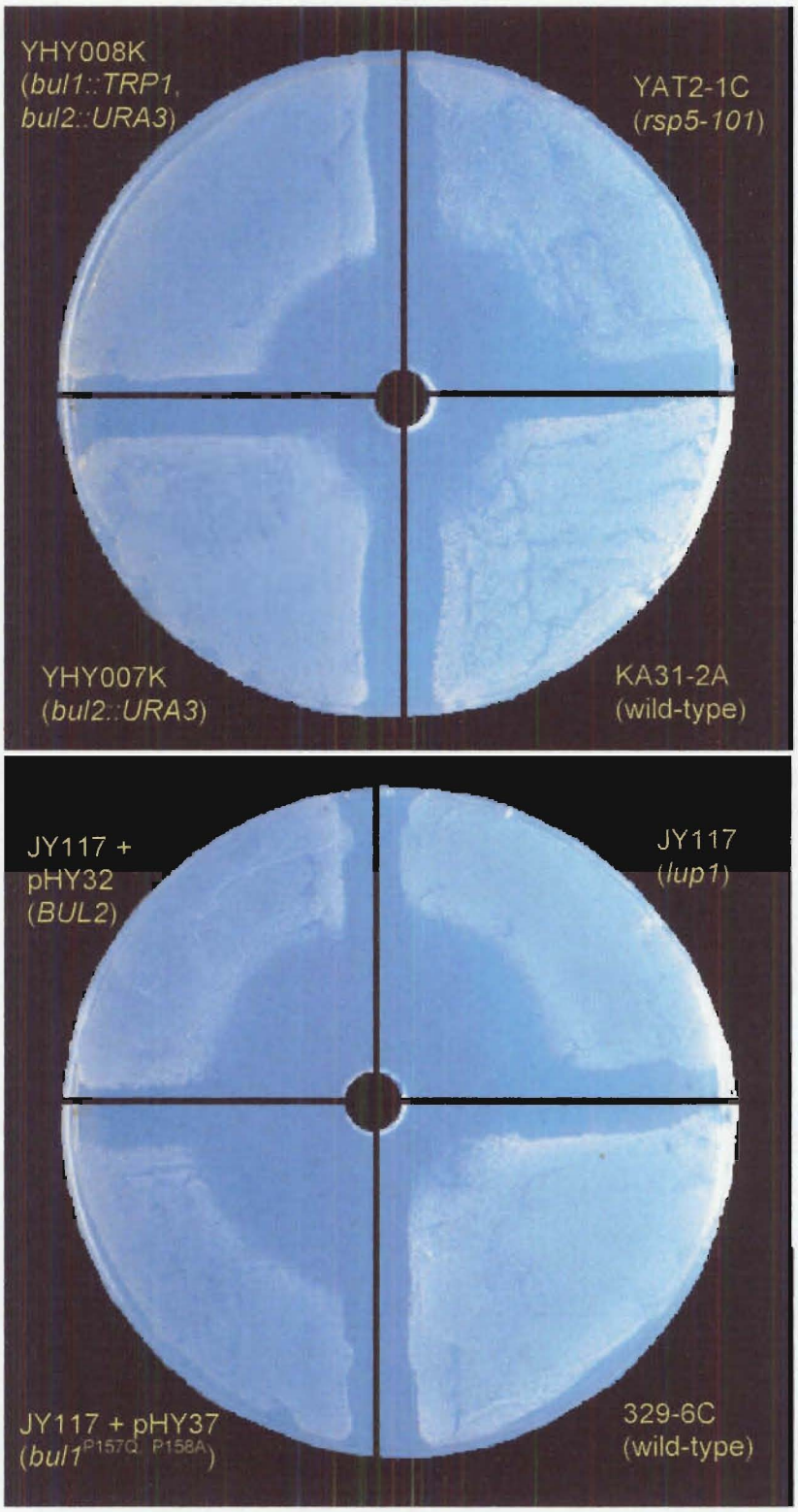

Figure 3.14: PGAs comparing sensitivity of various strains to *m*-fluoro-D,L-phenylalanine. Assays were carried out on SD + 0.5 HULAT medium and incubated for three days at 30°C.

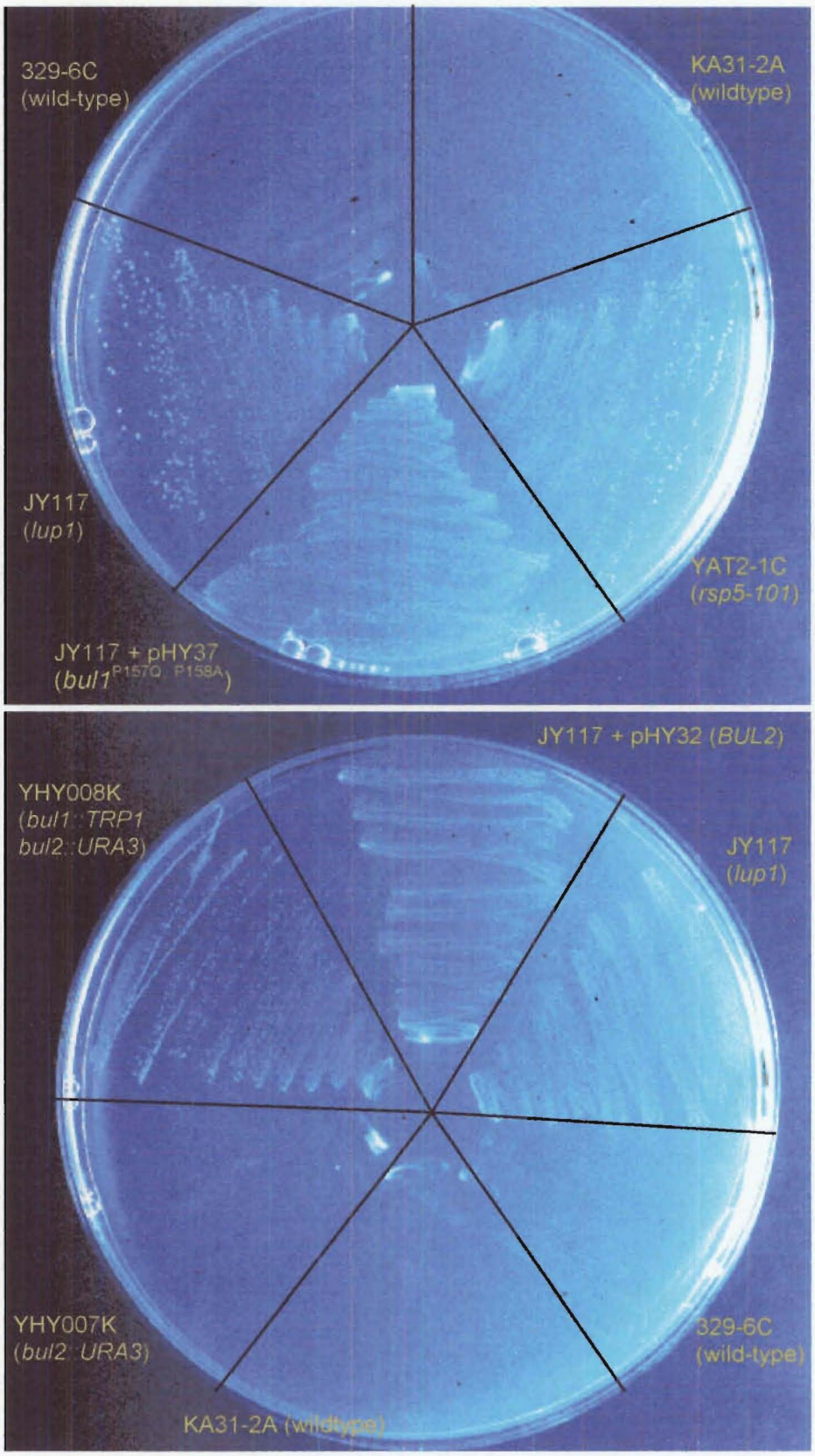

Figure 3.15: Comparison of ability of various strains to grow on LLM. LLM consists of 2.5 mg L^{-1} leucine incorporated into SC – LEU. Plates were incubated for three days at 30°C.

Table 3.6: Results of PGAs comparing sensitivities of various strains to *m*-fluoro-D,L-phenylalanine. Medium consisted of SD + 0.5 HULAT. The amount of *m*-fluoro-D,L-phenylalanine added to each plate included 200 μg. Results are expressed as ratios of distances of growth inhibition from the plate centre for Column A:Column B after three days of incubation at 30°C.

Column A	Column B	Average Ratio of Zones of Inhibition From Plate Centre for Column A:Column B
JY117 (*lup1*)	JY117 + pJO13	1.68 ± 0.05
329-6C (wildtype)	JY117 + pJO13	1
JY117 (*lup1*) + YCp50	JY117 + pJO13	1.68 + 0.05
JY117 (*lup1*) + pHY37 (pRS316-*bul1*$^{P157Q, P158A}$)	JY117 + pJO13	1.71 ± 0.08
JY117 (*lup1*) + pHY32 (pRS316-*BUL2*)	JY117 + pJO13	1.54 ± 0.07
YHY007K (*bul2::URA3*)	KA31-2A	0.86 ± 0.15
YHY008K (*bul1::TRP1 bul2::URA3*)	KA31-2A	1.69 ± 0.22
YAT2-1C (*rsp5-101*)	KA31-2A	1.88 ± 0.25
YAT2-1C (*rsp5-101*)	YAT2-1C + pHY08 (YCUp4-*RSP5*)	1.52 ± 0.09
YAT2-1C (*rsp5-101*) + YCp50	YAT2-1C + pHY08 (YCUp4-*RSP5*)	1.52 ± 0.09

Table 3.7: Results of MIC of *m*-fluoro-D,L-phenylalanine and minimum concentration of leucine for growth of various strains. SD + 0.5 HULAT medium was used for MIC determinations. Minimum concentrations of leucine were measured using SC – LEU medium for strains not containing plasmids and SC – LEU – URA for strains containing plasmids. Results were recorded after three days of incubation at 30°C.

Strain	MIC of *m*-fluoro-D,L-phenylalanine (mg L^{-1})	Minimum concentration of leucine for growth (mg L^{-1})
329-6C (wildtype)	30-40	10
JY117 (*lup1*)	10-15	2.5
JY117 (*lup1*) + YCp50	10-15	2.5
JY117 (*lup1*) + pJO13 (YCp50-*BUL1*)	30	10
JY117 (*lup1*) + pJO17 (YEp24-*BUL1*)	25-30	5
JY117 (*lup1*) + pJO24 (YEp24-*BUL1*)	20	5
JY117 (*lup1*) + pJO21 (YEp24-*ARO4*)	70	2.5
JY117 (*lup1*) + pHY37 (pRS316-*bul1*$^{P157Q, P158A}$)	10-15	2.5
JY117 (*lup1*) + pHY32 (pRS316-*BUL2*)	15	2.5
KA31-2A (wildtype)	60	7.5
YHY007K (*bul2::URA3*)	60	7.5
YHY008K (*bul1::TRP1 bul2::URA3*)	20-30	2.5
YAT2-1C (*rsp5-101*)	10-15	2.5
YAT2-1C (*rsp5-101*) + YCp50	10-15	5
YAT2-1C (*rsp5-101*) + pHY08 (YCUp4-*RSP5*)	20	5

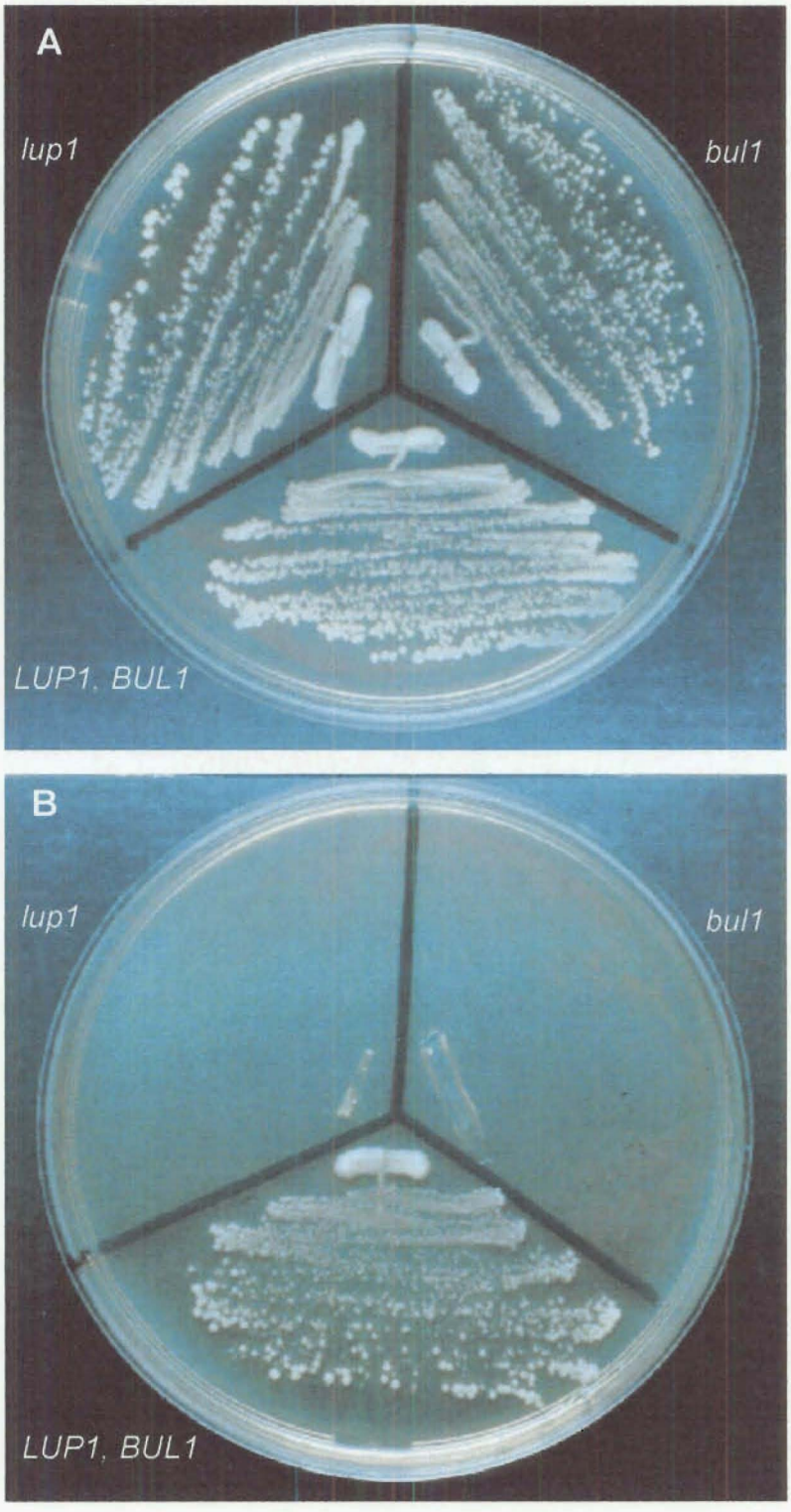

Figure 3.16: Comparison of the ability of JY117 (*lup1*), 329-6C (*LUP1*) and JOY64 (*bul1*) to grow on YPD medium at 30°C (A) and 37°C (B), after incubation for two days.

3.3.3 Temperature Sensitivity of *bul1* and *lup1* Mutants

Yashiroda et al. (1996) have described *bul1* mutants as temperature sensitive, whereas *bul2* disruptants were not (Yashiroda et al., 1998). *BUL1* and *bul1* strains can both grow at 30°C, whilst only wildtype yeast can grow at 37°C. The ability to grow at 30°C and 37°C was therefore compared for the *LUP1*, *lup1* and *bul1* strains, 329-6C, JY117 and JOY64, respectively. Strains were streaked onto YPD plates and incubated at these temperatures for two days. All strains formed similar sized colonies at 30°C, however, only 329-6C formed colonies at 37°C (Figure 3.16).

3.3.4 *BUL1/LUP1* Linkage Analysis

Demonstration that the associated functions and chromosomal positions of *BUL1* and *LUP1* overlap would provide convincing evidence that they were allelic. To investigate whether *BUL1* and *LUP1* occupy the same chromosomal position, analyses of the phenotypes of offspring of diploids created from mating a *lup1* strain with a strain containing a *BUL1* null allele were carried out. Figure 3.17 compares diploid and offspring phenotypes expected if *LUP1* and *BUL1* are separate alleles (Scenario 1) or the same allele (Scenario 2). Scenario 1 predicts that one out of four offspring resulting from sporulation of a *LUP1/lup1, BUL1/bul1* diploid will be of the Lup^- phenotype, assuming the two alleles are unlinked. Scenario 2 predicts that the *lup1/bul1, lup1/bul1* diploid created will give rise to 100% Lup^+ offspring after sporulation.

A partial knockout of the chromosomally encoded *BUL1* gene within SY1229 was created. Replacement of the *BUL1* allele was confirmed by restriction analyses of the PCR-amplified *BUL1* region, as described previously (Section 3.3.1). This strain, JOY74, was mated with a spontaneously arising His^+ variant of JY117 (JOY73). Diploids resulting from the cross were selected on SC-HIS-URA, and were assayed for Fpa^R and Lup status. A purified diploid, JOY77, was sporulated and spores were plated onto YPD to regenerate. 1083 resulting colonies were tested for Fpa, Lup, Ura and His phenotypes.

JOY77 was found to be Lup^+ and Fpa^S, as were all offspring (Table 3.8). The distance in centiMorgans (cM) between two genes, if there is a maximum of one exchange within the gene interval during meiosis, is equal to half the percentage of tetratype asci (asci in which one exchange had occurred between the two genes) following sporulation (Sherman and Wakem, 1991). An exchange between the *LUP1/lup1* and *BUL1/bul1* alleles, during meiosis of JOY77, would be predicted to result in a tetratype ascus containing one Fpa^R, Lup^- daughter. Assuming that out of the spores tested (equivalent to 1083/4 tetrads), one Fpa^R, Lup^- offspring was detected, *LUP1* and *BUL1* would be predicted to be approximately 0.18 cM apart (assuming that the Fpa^R and Lup^- phenotypes were not a result of spontaneous reversion). As no evidence of tetratype asci was detected, *LUP1* and *BUL1* are therefore within 0.18 cM. Ura and His phenotypes were used as controls. Assuming normal segregation is occurring, one would predict approximately 50% of offspring to be Ura^+ and the same for His^+, with approximately 25% being Ura^+ and His^+. We observed values similar to these (Table 3.8). Therefore, it was concluded that *LUP1* and *BUL1* were the same gene.

Table 3.8: Results of random spore analysis.

Phenotype	No. of Offspring Displaying Phenotype (total offspring)	Percentage of Offspring Displaying Phenotype (%)
Lup^+	1083 (1083)	100
Fpa^R	0 (1083)	0
Ura^+	597 (1083)	55.1
His^+	564 (1083)	52.1
Ura^+/His^+	295 (989)	29.8

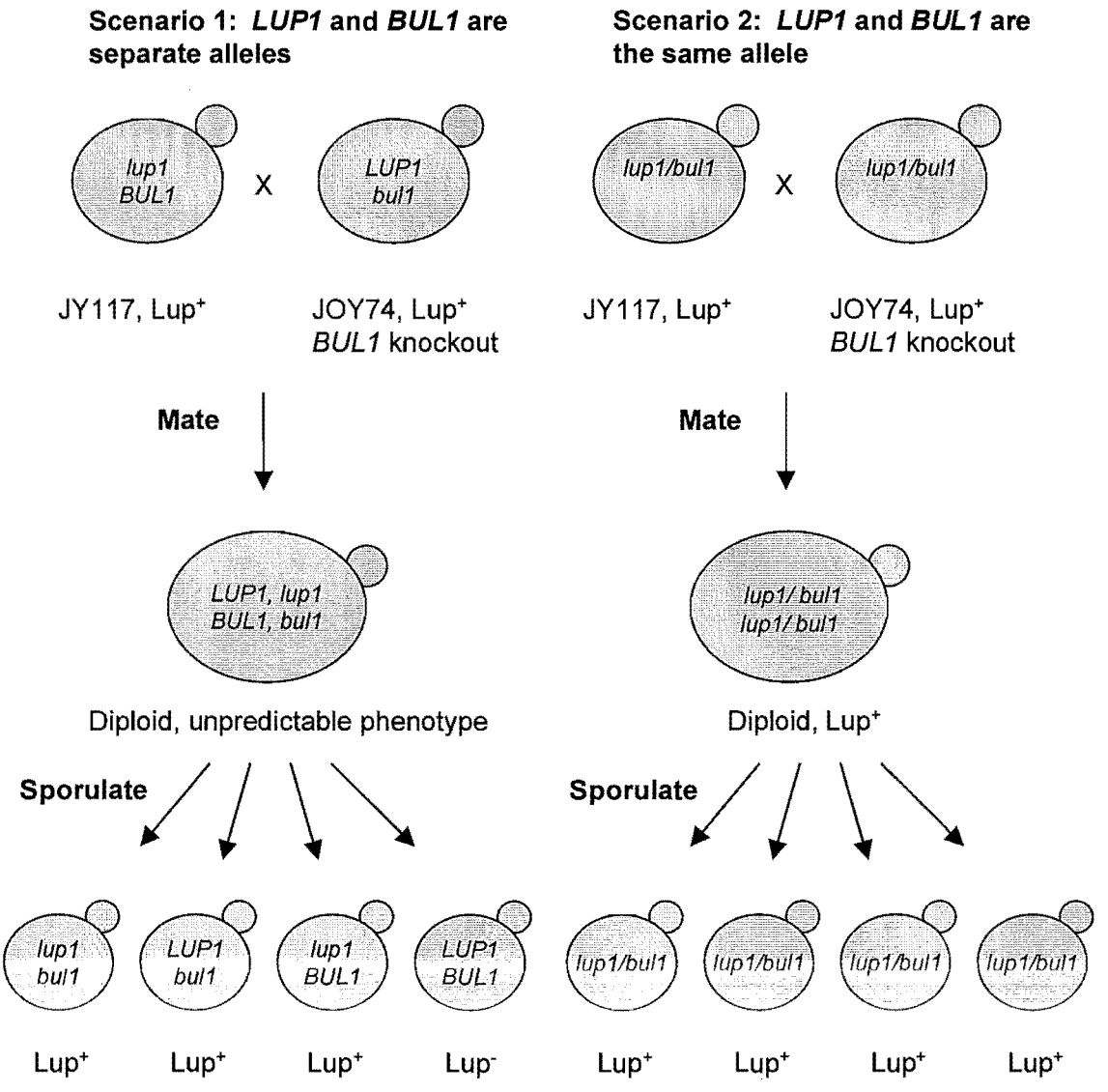

Figure 3.17: Predicted phenotypes of a diploid resulting from mating a *lup1* mutant and a *BUL1* knockout mutant; and the diploid offspring.
Scenario 1 assumes that *LUP1* and *BUL1* are separate alleles. Mating results in a diploid of unknown phenotypes with respect to Lup status, and assuming that *LUP1* and *BUL1* alleles are unlinked, one out of four offspring are predicted to be Lup^-.
Scenario 2 assumes that *LUP1* and *BUL1* are allelic. Mating results in a Lup^+ diploid, and 100% of offspring are expected to be Lup^+.

3.4 IS RSP5 FUNCTION AND INTERACTION WITH LUP1/BUL1 REQUIRED FOR LUP PERMEASE REGULATION?

Lup1/Bul1 has been proposed to function in association with the Rsp5 ubiquitin ligase as an E3 complex, involved in recognition and ubiquitination of specific proteins, thereby targeting them for degradation. A PY-motif (FPPSY) at the N-terminus of Lup1/Bul1 is required for physical interaction with Rsp5, and is essential for growth in various stress conditions (Yashiroda et al., 1998). If Rsp5 function and interaction with Lup1/Bul1 is required to mediate negative regulation of the Lup permease in the presence of ammonium, we would predict that viable *rsp5* mutants are also Lup^+, and that Lup1/Bul1 containing an altered PY-motif would be incapable of complementing the Lup^+ phenotypes of a *bul1* mutant.

To test whether Rsp5 function is required for regulation of the Lup permease, phenotypes of the ts *rsp5-101* mutant, YAT2-1C (Yashiroda et al., 1996), were compared with its cognate background, KA31-2A. In addition, YAT2-1C containing the low copy number plasmid YCp50 was compared with YAT2-1C carrying the low copy number plasmid pHY08. pHY08 encodes *RSP5* and complements the ts phenotype of YAT2-1C. The sensitivity of these strains to *m*-fluoro-D,L-phenylalanine was investigated using PGAs and MIC determinations. The lowest concentration of leucine capable of supporting growth of the strains was also determined.

PGAs indicated that YAT2-1C was more sensitive to *m*-fluoro-D,L-phenylalanine than its cognate wildtype (Table 3.6; Figure 3.14). Indeed, the MIC (Table 3.7) of the *rsp5-101* mutant was only 10-15 mg L^{-1} of *m*-fluoro-D,L-phenylalanine, compared with 60 mg L^{-1} for KA31-2A. YAT2-1C containing YCp50 was also more sensitive to *m*-fluoro-D,L-phenylalanine than YAT2-1C containing pHY08, although to a far lesser degree as the MIC for the latter was 20 mg mL^{-1}. Like JY117, YAT2-1C could form colonies when only 2.5 mg L^{-1} of leucine was present in SC - LEU medium, although KA31-2A required at least 7.5 mg L^{-1}. However, the lowest leucine concentrations that YAT2-1C containing YCp50 and YAT2-1C containing pHY08 could form colonies on were identical. Therefore, like disruption of *LUP1/BUL1*, the *rsp5-101* mutation also results in yeast portraying the Fpa^S phenotype, although not necessarily the Lup^+ phenotype.

These results do not rule out the involvement of *RSP5* in the regulation of the hypothetical Lup permease. YAT2-1C containing pHY08 would be expected to be a better control for this experiment than KA31-2A, as although KA31-2A is a cognate background, it is not isogenic. It is possible, however, that even though pHY08 is a single copy plasmid, *RSP5* expression from this plasmid may not emulate expression of *RSP5* from a wildtype cell. In addition, the mutation giving rise to the *rsp5-101* allele may not affect the ability of *RSP5* to regulate the hypothetical Lup permease. This discrepancy could be resolved by studying the phenotypes of other *rsp5* mutants.

To investigate whether interaction between Rsp5 and Lup1/Bul1 is required for Lup permease regulation, the ability of the single copy plasmid pHY37 to complement phenotypes of a Lup^+ mutant was determined. pHY37 carries $bul1^{P157Q,\ P158A}$, which encodes Lup1/Bul1 with a PY motif altered from PPSY to QASY (Yashiroda et al., 1998). pHY37 was introduced into JY117. Sensitivity to *m*-fluoro-D,L-phenylalanine of 329-6C, JY117 and JY117 containing pHY37, was compared using PGAs and MIC determinations. The lowest concentration of leucine that these strains could grow on was also determined.

PGA (Table 3.6; Figure 3.14) and MIC results (Table 3.7) showed that sensitivity of JY117 to *m*-fluoro-D,L-phenylalanine was not decreased when pHY37 was present. Moreover, the presence of pHY37 did not increase the minimum leucine concentration required by JY117, thus expression of $bul1^{P157Q,\ P158A}$ does not decrease the leucine-scavenging ability of JY117 (Table 3.7, Figure 3.15). The inability of $bul1^{P157Q,\ P158A}$ to complement the Lup^+ and Fpa^S phenotypes of JY117 therefore indicates that the two proline residues within the PPSY motif of Lup1/Bul1 are essential for Lup1/Bul1 regulation of the Lup permease. Since Rsp5 may also be involved in regulation of the Lup permease, the inability of $Bul1^{P157Q,\ P158A}$ to regulate the Lup permease may be because it cannot interact with Rsp5. However, we cannot at this time distinguish between an interaction between proteins mediated by the proline residues and other effects of the mutation on Lup1 function.

3.5 *BUL1/LUP1* SEQUENCE OF LUP$^+$ MUTANTS AND 329-6C

Heinemann et al. (1994) have reported an unusually high mutation rate of wildtype to the Lup$^+$ phenotype of approximately 1 variant per 10^6 input cells. Furthermore, this phenotype was eventually displayed by a large proportion of the population when grown on selective media (approximately 1 per 100 input cells in 12 days). The cause is not known, although it is possible that DNA metabolism continued during leucine starvation. Determining the DNA sequence of *lup1* may provide an explanation for the high mutation rate, for example the *BUL1/LUP1* region may be highly susceptible to recombination or transposon insertion. To this end, the *BUL1/LUP1* sequences of wildtype 329-6C and two spontaneously arising Lup$^+$ mutants (JY117 and JOY53) were established, and compared to the published *BUL1/LUP1* sequence.

The Lup$^+$ mutants were independently isolated following plating of 329-6C on LLM. JY117 was isolated by Heinemann et al. (1994) and JOY53 was isolated in this study. The Lup$^+$ and Fpa^S phenotypes of both mutants could be complemented by the expression of plasmid-bourne *BUL1/LUP1*. A series of approximately 800 bp-sized regions spanning *BUL1/LUP1* or *bul1/lup1* were amplified by PCR from genomic DNA isolated from the three strains, using primer pairs JO1 and JO2, JO3 and JO4, JO5 and JO6, and JO7 and JO8 (Appendix 3). As amplification of DNA by the Expand™ High Fidelity PCR System has a small misincorporation rate ($8.5x10^{-6}$), pools of amplified product were sequenced directly, rather than sequencing an individual DNA molecule cloned from this pool. When discrepancies occurred between DNA sequences from different strains, the region was reamplified and resequenced.

A comparison between the *LUP1* sequence and published *BUL1*, *ZZZ1* and *RDS1* sequences is outlined in Figure 3.20. Interestingly, there were 27 nucleotide differences between the reported *BUL1* sequence and the *BUL1/LUP1* sequence of our wildtype, 329-6C. These nucleotide differences corresponded to nine amino acid changes. Changes represented 0.9% of *BUL1/LUP1* nucleotide and amino acid sequence. The *BUL1/LUP1* sequence of 329-6C also differed from *ZZZ1* and *RDS1* sequences. In addition to the amino acid differences between Bul1/Lup1 and the published Bul1 sequence, Zzz1 contained a valine at amino acid position 35 whereas Bul1/Lup1 contained an alanine; and Rds1 contained an arginine at amino acid position 963

whereas an alanine comprised this position in Bul1/Lup1. The *bul1/lup1* sequence from JY117 was identical to *BUL1/LUP1* from 329-6C, except for a deletion of the nucleotide guanine (G) at position 2010 (Figure 3.18), resulting in a frameshift mutation. The deleted base corresponded to a base that was different between the published *BUL1* sequence and that of 329-6C. The *bul1/lup1* sequence from the second spontaneously arising mutant, JOY53, was also identical to 329-6C, except at one nucleotide. At nucleotide number 1008, a transversion event occurred, resulting in the replacement of cytosine (C) by adenine (A) (Figure 3.19), and corresponding to a change in amino acid from serine (S) to arginine (R). Elucidation of the nature of mutation in *BUL1/LUP1* in mutants JY117 and JOY53 therefore does not appear to offer any explanation as to the cause of the unusually high mutation rate.

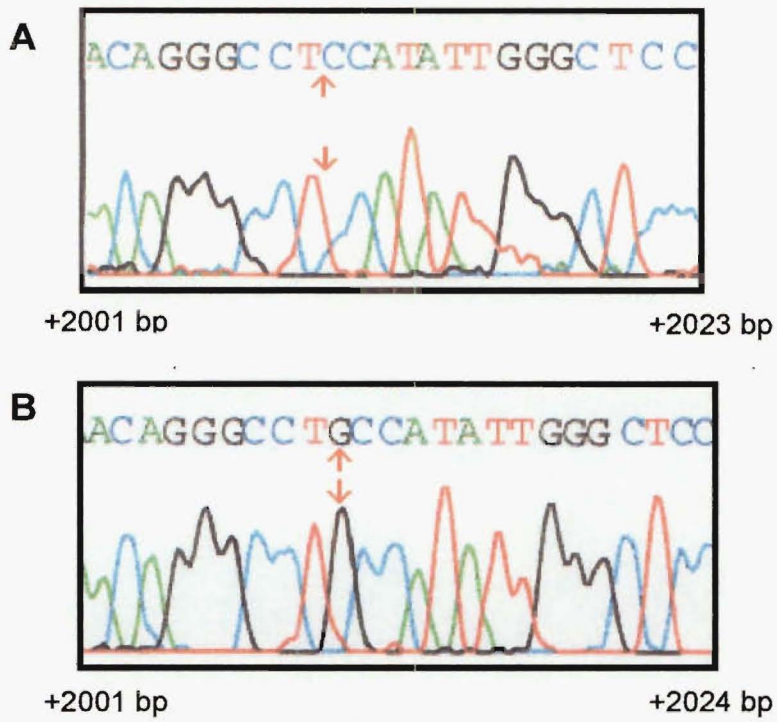

Figure 3.18: Electropherograms of *BUL1/LUP1* coding sequence from 329-6C (B) and JY117 (A). Electropherograms demonstrate deletion of a guanine (G) residue at position 2010 in JY117 sequence.

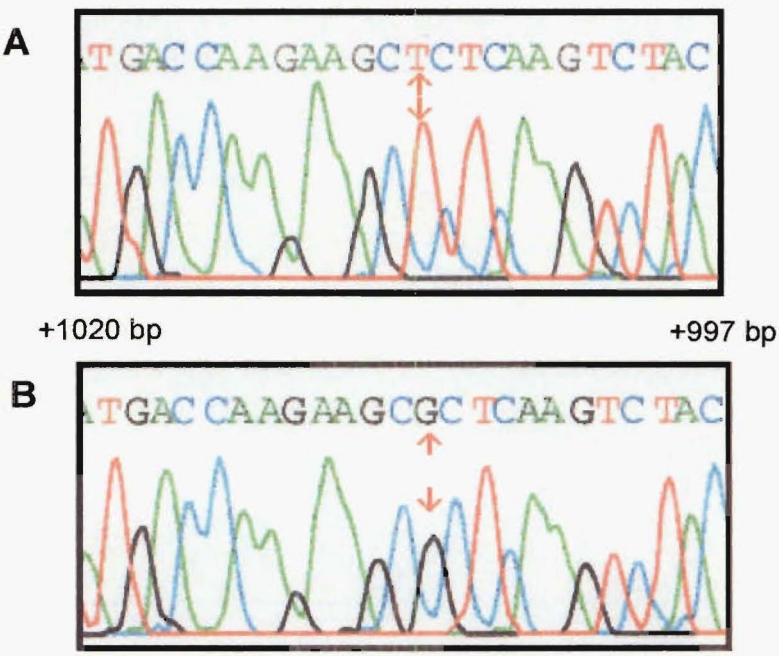

Figure 3.19: Electropherograms of *BUL1/LUP1* coding sequence from JOY53 (A) and 329-6C (B). A guanine (G) residue has been replaced by a thymine (T) residue at position 1008 in sequence from JOY53.

Figure 3.20: Comparison of nucleotide and amino acid sequences of the alleles *BUL1*, *RDS1*, *ZZZ1* and *LUP1*. The GenBank database accession numbers for the sequences include D50083 (*BUL1*), *ZZZ1* (Wolfe et al., 1999) and X88901 (*RDS1*). Where sequence differs from that of *BUL1* or Bul1, the background of the altered base or amino acid is shaded black. The bases mutated in the *lup1* strains JY117 and JOY53 are indicated by arrows. *BUL1* bases that are shaded grey represent sequence identical to sequence information obtained for pJO34.

```
BUL1:  -60  tcttagcaagggcgaaaagagactgttcgtgtgtgtgtcaacaggtatattgtacgctaa
RDS1:  -60  tcttagcaagggcgaaaagagactgttcgtgtgtgtgtcaacaggtatattgtacgctaa
ZZZ1:  -60  tcttagcaagggcgaaaagagactgttcgtgtgtgtgtcaacaggtatattgtacgctaa
LUP1:  -60  tcttagcaagggcgaaaagagactgttcgtgtgtgtgtcaacaggtatattgtacgctaa

BUL1:    1  atggccaaagatttgaacgattcggggtttccaccgaagaggaagcctttgctgcgtcct
         1   M  A  K  D  L  N  D  S  G  F  P  P  K  R  K  P  L  L  R  P
RDS1:    1  atggccaaagatttgaacgattcggggtttccaccgaagaggaagcctttgctgcgtcct
         1   M  A  K  D  L  N  D  S  G  F  P  P  K  R  K  P  L  L  R  P
ZZZ1:    1  atggccaaagatttgaacgattcggggtttccaccgaagaggaagcctttgctgcgtcct
         1   M  A  K  D  L  N  D  S  G  F  P  P  K  R  K  P  L  L  R  P
LUP1:    1  atggccaaagatttgaacgattcggggtttccaccgaagaggaagcctttgctgcgtcct
         1   M  A  K  D  L  N  D  S  G  F  P  P  K  R  K  P  L  L  R  P

BUL1:   61  caacgatctgattttactgcaaatagttcgacaactatgaacgctaatgcaaacacaagg
        21   Q  R  S  D  F  T  A  N  S  S  T  T  M  N  A  N  A  N  T  R
RDS1:   61  caacgatctgattttaccgcaaatagttcgacaactatgaacgttaatgcaaacacaagg
        21   Q  R  S  D  F  T  A  N  S  S  T  T  M  N  V  N  A  N  T  R
ZZZ1:   61  caacgatctgattttaccgcaaatagttcgacaactatgaacgttaatgcaaacacaagg
        21   Q  R  S  D  F  T  A  N  S  S  T  T  M  N  V  N  A  N  T  R
LUP1:   61  caacgatctgattttactgcaaatagttcgacaactatgaacgctaatgcaaacacaagg
        21   Q  R  S  D  F  T  A  N  S  S  T  T  M  N  A  N  A  N  T  R

BUL1:  121  gggcgtggtaggcagaaacaagagggtggtaaaggcagttcgaggtcaccgtcgttacac
        41   G  R  G  R  Q  K  Q  E  G  G  K  G  S  S  R  S  P  S  L  H
RDS1:  121  gggcgtggtaggcagaaacaagagggtggtaaaggcagttcgaggtcaccgtcgttacac
        41   G  R  G  R  Q  K  Q  E  G  G  K  G  S  S  R  S  P  S  L  H
ZZZ1:  121  gggcgtggtaggcagaaacaagagggtggtaaaggcagttcgaggtcaccgtcgttacac
        41   G  R  G  R  Q  K  Q  E  G  G  K  G  S  S  R  S  P  S  L  H
LUP1:  121  gggcgtggtaggcagaaacaagagggtggtaaaggcagttcgaggtcaccgtcgttacac
        41   G  R  G  R  Q  K  Q  E  G  G  K  G  S  S  R  S  P  S  L  H

BUL1:  181  tctccaaaatcatggataagaagcgcgtctgctacggggatccttggactaagacgtccc
        61   S  P  K  S  W  I  R  S  A  S  A  T  G  I  L  G  L  R  R  P
RDS1:  181  tctccaaaatcatggataagaagcgcgtctgctacggggatccttggactaagacgtccc
        61   S  P  K  S  W  I  R  S  A  S  A  T  G  I  L  G  L  R  R  P
ZZZ1:  181  tctccaaaatcatggataagaagcgcgtctgctacggggatccttggactaagacgtccc
        61   S  P  K  S  W  I  R  S  A  S  A  T  G  I  L  G  L  R  R  P
LUP1:  181  tctccaaaatcatggataagaagcgcgtctgctacggggattcttggactaagacgtccc
        61   S  P  K  S  W  I  R  S  A  S  A  T  G  I  L  G  L  R  R  P

BUL1:  241  gaactagcacattctcattcgcatgctccttccaccggaacgcctgctggaggtaatcgt
        81   E  L  A  H  S  H  S  H  A  P  S  T  G  T  P  A  G  G  N  R
RDS1:  241  gaactagcacattctcattcgcatgctccttccaccggaacgcctgctggaggtaatcgt
        81   E  L  A  H  S  H  S  H  A  P  S  T  G  T  P  A  G  G  N  R
ZZZ1:  241  gaactagcacattctcattcgcatgctccttccaccggaacgcctgctggaggtaatcgt
        81   E  L  A  H  S  H  S  H  A  P  S  T  G  T  P  A  G  G  N  R
LUP1:  241  gaactagcacattccattcgcatgctccttcaaccggaacgcctgctggaggtaatcgt
        81   E  L  A  H  S  H  S  H  A  P  S  T  G  T  P  A  G  G  N  R
```

Figure 3.20: continued

```
BUL1:   301 tctcctttgagaagatcaactgcaaatgcgacccccgtcgagaccggaaggtccttgacc
        101  S  P  L  R  R  S  T  A  N  A  T  P  V  E  T  G  R  S  L  T
RDS1:   301 tctcctttgagaagatcaactgcaaatgcgacccccgtcgagaccggaaggtccttgacc
        101  S  P  L  R  R  S  T  A  N  A  T  P  V  E  T  G  R  S  L  T
ZZZ1:   301 tctcctttgagaagatcaactgcaaatgcgacccccgtcgagaccggaaggtccttgacc
        101  S  P  L  R  R  S  T  A  N  A  T  P  V  E  T  G  R  S  L  T
LUP1:   301 tctcctttgagaagatcaactgcaaatgcgacccccgtcgagaccgaaaggtccttgacc
        101  S  P  L  R  R  S  T  A  N  A  T  P  V  E  T  E  R  S  L  T

BUL1:   361 gacggagatatcaacaatgttgttgatgtactgccctcatttgagatgtacaataccctg
        121  D  G  D  I  N  N  V  V  D  V  L  P  S  F  E  M  Y  N  T  L
RDS1:   361 gacggagatatcaacaatgttgttgatgtactgccctcatttgagatgtacaataccctg
        121  D  G  D  I  N  N  V  V  D  V  L  P  S  F  E  M  Y  N  T  L
ZZZ1:   361 gacggagatatcaacaatgttgttgatgtactgccctcatttgagatgtacaataccctg
        121  D  G  D  I  N  N  V  V  D  V  L  P  S  F  E  M  Y  N  T  L
LUP1:   361 gacggagatatcaacaacgttgttgatgtactgccctcatttgagatgtacaatgctctg
        121  D  G  D  I  N  N  V  V  D  V  L  P  S  F  E  M  Y  N  T  L

BUL1:   421 cataggcacattccgcagggcaacgtcgatccagataggcatgatttcccaccttcatac
        141  H  R  H  I  P  Q  G  N  V  D  P  D  R  H  D  F  P  P  S  Y
RDS1:   421 cataggcacattccgcagggcaacgtcgatccagataggcatgatttcccaccttcatac
        141  H  R  H  I  P  Q  G  N  V  D  P  D  R  H  D  F  P  P  S  Y
ZZZ1:   421 cataggcacattccgcagggcaacgtcgatccagataggcatgatttcccaccttcatac
        141  H  R  H  I  P  Q  G  N  V  D  P  D  R  H  D  F  P  P  S  Y
LUP1:   421 cataggcacattccgcagggcaacgtcgatccagataggcatgatttcccaccttcatac
        141  H  R  H  I  P  Q  G  N  V  D  P  D  R  H  D  F  P  P  S  Y

BUL1:   481 caagaggccaataattctactgcaacaggtgctgcgggctcgagcgctgatctctcacat
        161  Q  E  A  N  N  S  T  A  T  G  A  A  G  S  S  A  D  L  S  H
RDS1:   481 caagaggccaataattctactgcaacaggtgctgcgggctcgagcgctgatctctcacat
        161  Q  E  A  N  N  S  T  A  T  G  A  A  G  S  S  A  D  L  S  H
ZZZ1:   481 caagaggccaataattctactgcaacaggtgctgcgggctcgagcgctgatctctcacat
        161  Q  E  A  N  N  S  T  A  T  G  A  A  G  S  S  A  D  L  S  H
LUP1:   481 caagaggccaataattctactgcaacaggtgctgcgggctcgagcgctgatctctcacat
        161  Q  E  A  N  N  S  T  A  T  G  A  A  G  S  S  A  D  L  S  H

BUL1:   541 caatcattgtccactgacgcattgggtgccacacgttcttcgtcaacatcaaatttagaa
        181  Q  S  L  S  T  D  A  L  G  A  T  R  S  S  S  T  S  N  L  E
RDS1:   541 caatcattgtccactgacgcattgggtgccacacgttcttcgtcaacatcaaatttagaa
        181  Q  S  L  S  T  D  A  L  G  A  T  R  S  S  S  T  S  N  L  E
ZZZ1:   541 caatcattgtccactgacgcattgggtgccacacgttcttcgtcaacatcaaatttagaa
        181  Q  S  L  S  T  D  A  L  G  A  T  R  S  S  S  T  S  N  L  E
LUP1:   541 caatcattgtccactgacgcattgggtgccacacgttcttcgtcaacatcaaatttagaa
        181  Q  S  L  S  T  D  A  L  G  A  T  R  S  S  S  T  S  N  L  E

BUL1:   601 aacttaattcccccttcgaaccgaacatcacagtattgcagcacatcaatcaaccgctgtc
        201  N  L  I  P  L  R  T  E  H  H  S  I  A  A  H  Q  S  T  A  V
RDS1:   601 aacttaattcccccttcgaaccgaacatcacagtattgcagcacatcaatcaaccgctgtc
        201  N  L  I  P  L  R  T  E  H  H  S  I  A  A  H  Q  S  T  A  V
ZZZ1:   601 aacttaattcccccttcgaaccgaacatcacagtattgcagcacatcaatcaaccgctgtc
        201  N  L  I  P  L  R  T  E  H  H  S  I  A  A  H  Q  S  T  A  V
LUP1:   601 aacttaattcccccttcgaaccgaacatcacagtattgcagcacatcaatcaaccgctgtc
        201  N  L  I  P  L  R  T  E  H  H  S  I  A  A  H  Q  S  T  A  V

BUL1:   661 gatgaagattcactggatatacctcccatacttgatgacttgaacgatacagacaacatt
        221  D  E  D  S  L  D  I  P  P  I  L  D  D  L  N  D  T  D  N  I
RDS1:   661 gatgaagattcactggatatacctcccatacttgatgacttgaacgatacagacaacatt
        221  D  E  D  S  L  D  I  P  P  I  L  D  D  L  N  D  T  D  N  I
```

Figure 3.20: continued

```
ZZZ1:   661 gatgaagattcactggatatacctcccatacttgatgacttgaacgatacagacaacatt
        221  D  E  D  S  L  D  I  P  P  I  L  D  D  L  N  D  T  D  N  I
LUP1:   661 gatgaagattcactggatgtacctcccatacttgatgacttgaacgatacagacaacatt
        221  D  E  D  S  L  D  V  P  P  I  L  D  D  L  N  D  T  D  N  I

BUL1:   721 ttcatcgacaaattgtacactttaccaaaaatgtccacacccatcgaaatcaccatcaag
        241  F  I  D  K  L  Y  T  L  P  K  M  S  T  P  I  E  I  T  I  K
RDS1:   721 ttcatcgacaaattgtacactttaccaaaaatgtccacacccatcgaaatcaccatcaag
        241  F  I  D  K  L  Y  T  L  P  K  M  S  T  P  I  E  I  T  I  K
ZZZ1:   721 ttcatcgacaaattgtacactttaccaaaaatgtccacacccatcgaaatcaccatcaag
        241  F  I  D  K  L  Y  T  L  P  K  M  S  T  P  I  E  I  T  I  K
LUP1:   721 ttcatcgacaaattgtacactttaccaaaaatgtccacacccatcgaaatcaccatcaag
        241  F  I  D  K  L  Y  T  L  P  K  M  S  T  P  I  E  I  T  I  K

BUL1:   781 acgacgaagcatgcacctataccacacgtgaagccggaggaggagtccattttgaaagag
        261  T  T  K  H  A  P  I  P  H  V  K  P  E  E  E  S  I  L  K  E
RDS1:   781 acgacgaagcatgcacctataccacacgtgaagccggaggaggagtccattttgaaagag
        261  T  T  K  H  A  P  I  P  H  V  K  P  E  E  E  S  I  L  K  E
ZZZ1:   781 acgacgaagcatgcacctataccacacgtgaagccggaggaggagtccattttgaaagag
        261  T  T  K  H  A  P  I  P  H  V  K  P  E  E  E  S  I  L  K  E
LUP1:   781 acgacgaagcatgcacctataccacacgtgaagccggaggaggagtccattttgaaagag
        261  T  T  K  H  A  P  I  P  H  V  K  P  E  E  E  S  I  L  K  E

BUL1:   841 tatacgtcgggggatttgattcatggttttatcactattgaaaacaaatctcaagcaaac
        281  Y  T  S  G  D  L  I  H  G  F  I  T  I  E  N  K  S  Q  A  N
RDS1:   841 tatacgtcgggggatttgattcatggttttatcactattgaaaacaaatctcaagcaaac
        281  Y  T  S  G  D  L  I  H  G  F  I  T  I  E  N  K  S  Q  A  N
ZZZ1:   841 tatacgtcgggggatttgattcatggttttatcactattgaaaacaaatctcaagcaaac
        281  Y  T  S  G  D  L  I  H  G  F  I  T  I  E  N  K  S  Q  A  N
LUP1:   841 tatacgtcgggggatttgattcatggttttatcacgattgaaaacaaatctcaagcaaac
        281  Y  T  S  G  D  L  I  H  G  F  I  T  I  E  N  K  S  Q  A  N

BUL1:   901 ctaaagtttgaaatgttctatgtcactttagagtcttacatttccattattgataaagta
        301  L  K  F  E  M  F  Y  V  T  L  E  S  Y  I  S  I  I  D  K  V
RDS1:   901 ctaaagtttgaaatgttctatgtcactttagagtcttacatttccattattgataaagta
        301  L  K  F  E  M  F  Y  V  T  L  E  S  Y  I  S  I  I  D  K  V
ZZZ1:   901 ctaaagtttgaaatgttctatgtcactttagagtcttacatttccattattgataaagta
        301  L  K  F  E  M  F  Y  V  T  L  E  S  Y  I  S  I  I  D  K  V
LUP1:   901 ctaaagtttgaaatgttctatgtcactttagagtcttacatttccattattgataaagta
        301  L  K  F  E  M  F  Y  V  T  L  E  S  Y  I  S  I  I  D  K  V

BUL1:   961 aagagtaaaagaacgattaaacggtttttaaggatggtagacttgagcgcttcttggtca
        321  K  S  K  R  T  I  K  R  F  L  R  M  V  D  L  S  A  S  W  S
RDS1:   961 aagagtaaaagaacgattaaacggtttttaaggatggtagacttgagcgcttcttggtca
        321  K  S  K  R  T  I  K  R  F  L  R  M  V  D  L  S  A  S  W  S
ZZZ1:   961 aagagtaaaagaacgattaaacggtttttaaggatggtagacttgagcgcttcttggtca
        321  K  S  K  R  T  I  K  R  F  L  R  M  V  D  L  S  A  S  W  S
LUP1:   961 aagagtaaaagaacgattaaacggtttttaaggatggtagacttgagcgcttcttggtca
        321  K  S  K  R  T  I  K  R  F  L  R  M  V  D  L  S▲ A  S  W  S

BUL1:  1021 tactcgaaaatagcactggggtccggtgtggacttcattcccgcagatgttgactacgat
        341  Y  S  K  I  A  L  G  S  G  V  D  F  I  P  A  D  V  D  Y  D
RDS1:  1021 tactcgaaaatagcactggggtccggtgtggacttcattcccgcagatgttgactacgat
        341  Y  S  K  I  A  L  G  S  G  V  D  F  I  P  A  D  V  D  Y  D
ZZZ1:  1021 tactcgaaaatagcactggggtccggtgtggacttcattcccgcagatgttgactacgat
        341  Y  S  K  I  A  L  G  S  G  V  D  F  I  P  A  D  V  D  Y  D
LUP1:  1021 tactcgaaaatagcactggggtccggtgtggacttcattcctgcagatgttgactacgat
        341  Y  S  K  I  A  L  G  S  G  V  D  F  I  P  A  D  V  D  Y  D
```

Figure 3.20: continued

```
BUL1:  1081 ggttctgtatttgggctaaacaatagccgggttctggaacccggagtcaagtacaagaaa
        361  G  S  V  F  G  L  N  N  S  R  V  L  E  P  G  V  K  Y  K  K
RDS1:  1081 ggttctgtatttgggctaaacaatagccgggttctggaacccggagtcaagtacaagaaa
        361  G  S  V  F  G  L  N  N  S  R  V  L  E  P  G  V  K  Y  K  K
ZZZ1:  1081 ggttctgtatttgggctaaacaatagccgggttctggaacccggagtcaagtacaagaaa
        361  G  S  V  F  G  L  N  N  S  R  V  L  E  P  G  V  K  Y  K  K
LUP1:  1081 ggttctgtatttgggctaaacaatagccgggttctggaacccggggtcaagtacaagaaa
        361  G  S  V  F  G  L  N  N  S  R  V  L  E  P  G  V  K  Y  K  K

BUL1:  1141 ttcttcattttcaaattgccactgcaattgctagatgtcacttgtaagcaggagcatttc
        381  F  F  I  F  K  L  P  L  Q  L  L  D  V  T  C  K  Q  E  H  F
RDS1:  1141 ttcttcattttcaaattgccactgcaattgctagatgtcacttgtaagcaggagcatttc
        381  F  F  I  F  K  L  P  L  Q  L  L  D  V  T  C  K  Q  E  H  F
ZZZ1:  1141 ttcttcattttcaaattgccactgcaattgctagatgtcacttgtaagcaggagcatttc
        381  F  F  I  F  K  L  P  L  Q  L  L  D  V  T  C  K  Q  E  H  F
LUP1:  1141 ttcttcattttcaaattgccactgcaattgctagatgtcacttgtaagcaggagcatttc
        381  F  F  I  F  K  L  P  L  Q  L  L  D  V  T  C  K  Q  E  H  F

BUL1:  1201 tctcattgtttgttacctcccagttttggtattgataaatataggaacaattgcaaatat
        401  S  H  C  L  L  P  P  S  F  G  I  D  K  Y  R  N  N  C  K  Y
RDS1:  1201 tctcattgtttgttacctcccagttttggtattgataaatataggaacaattgcaaatat
        401  S  H  C  L  L  P  P  S  F  G  I  D  K  Y  R  N  N  C  K  Y
ZZZ1:  1201 tctcattgtttgttacctcccagttttggtattgataaatataggaacaattgcaaatat
        401  S  H  C  L  L  P  P  S  F  G  I  D  K  Y  R  N  N  C  K  Y
LUP1:  1201 tctcattgtttgtcacctcccagttttggtattgataaatataggaacaattgcaaatat
        401  S  H  C  L  S  P  P  S  F  G  I  D  K  Y  R  N  N  C  K  Y

BUL1:  1261 tccggtatcaaagtcaatagggtacttgggtgcggtcatttaggtacaaagggctccccc
        421  S  G  I  K  V  N  R  V  L  G  C  G  H  L  G  T  K  G  S  P
RDS1:  1261 tccggtatcaaagtcaatagggtacttgggtgcggtcatttaggtacaaagggctccccc
        421  S  G  I  K  V  N  R  V  L  G  C  G  H  L  G  T  K  G  S  P
ZZZ1:  1261 tccggtatcaaagtcaatagggtacttgggtgcggtcatttaggtacaaagggctccccc
        421  S  G  I  K  V  N  R  V  L  G  C  G  H  L  G  T  K  G  S  P
LUP1:  1261 tccggtatcaaagtcaatagttgtacttgggtgcggtcatttaggtacaaagggctccccc
        421  S  G  I  K  V  N  S  V  L  G  C  G  H  L  G  T  K  G  S  P

BUL1:  1321 atcttaactaacgatatgtctgatgacaacctttcgatcaattacactattgatgcaagg
        441  I  L  T  N  D  M  S  D  D  N  L  S  I  N  Y  T  I  D  A  R
RDS1:  1321 atcttaactaacgatatgtctgatgacaacctttcgatcaattacactattgatgcaagg
        441  I  L  T  N  D  M  S  D  D  N  L  S  I  N  Y  T  I  D  A  R
ZZZ1:  1321 atcttaactaacgatatgtctgatgacaacctttcgatcaattacactattgatgcaagg
        441  I  L  T  N  D  M  S  D  D  N  L  S  I  N  Y  T  I  D  A  R
LUP1:  1321 atcttaactaacgatatgtctgatgacaacctttcgatcaattacactattgatgcaagg
        441  I  L  T  N  D  M  S  D  D  N  L  S  I  N  Y  T  I  D  A  R

BUL1:  1381 attgtcggtaaagatcaaaaggcctctaaactgtatattatgaaggaaagagaatataat
        461  I  V  G  K  D  Q  K  A  S  K  L  Y  I  M  K  E  R  E  Y  N
RDS1:  1381 attgtcggtaaagatcaaaaggcctctaaactgtatattatgaaggaaagagaatataat
        461  I  V  G  K  D  Q  K  A  S  K  L  Y  I  M  K  E  R  E  Y  N
ZZZ1:  1381 attgtcggtaaagatcaaaaggcctctaaactgtatattatgaaggaaagagaatataat
        461  I  V  G  K  D  Q  K  A  S  K  L  Y  I  M  K  E  R  E  Y  N
LUP1:  1381 attgtcggtaaagatcaaaaggcctctaaactgtatattatgaaggaaagagaatataat
        461  I  V  G  K  D  Q  K  A  S  K  L  Y  I  M  K  E  R  E  Y  N

BUL1:  1441 ctaagagtaatccctttttggttttgacgccaatgtcgtcggagaaagaaccactatgagt
        481  L  R  V  I  P  F  G  F  D  A  N  V  V  G  E  R  T  T  M  S
RDS1:  1441 ctaagagtaatccctttttggttttgacgccaatgtcgtcggagaaagaaccactatgagt
        481  L  R  V  I  P  F  G  F  D  A  N  V  V  G  E  R  T  T  M  S
```

Figure 3.20: continued

```
ZZZ1:  1441 ctaagagtaatccctttttggttttgacgccaatgtcgtcggagaaagaaccactatgagt
        481  L  R  V  I  P  F  G  F  D  A  N  V  V  G  E  R  T  T  M  S
LUP1:  1441 ctaagagtaatccctttttggttttgacgccaatgtcgtcggagaaagaaccactatgagt
        481  L  R  V  I  P  F  G  F  D  A  N  V  V  G  E  R  T  T  M  S

BUL1:  1501 cagctgaatgatatcaccaaactagtgcaggaaaggttggatgctcttagaaaaatcttt
        501  Q  L  N  D  I  T  K  L  V  Q  E  R  L  D  A  L  R  K  I  F
RDS1:  1501 cagctgaatgatatcaccaaactagtgcaggaaaggttggatgctcttagaaaaatcttt
        501  Q  L  N  D  I  T  K  L  V  Q  E  R  L  D  A  L  R  K  I  F
ZZZ1:  1501 cagctgaatgatatcaccaaactagtgcaggaaaggttggatgctcttagaaaaatcttt
        501  Q  L  N  D  I  T  K  L  V  Q  E  R  L  D  A  L  R  K  I  F
LUP1:  1501 cagctgaatgatatcaccaaactagtgcaggaaaggttggatgctcttagaaaaatcttt
        501  Q  L  N  D  I  T  K  L  V  Q  E  R  L  D  A  L  R  K  I  F

BUL1:  1561 cagagattagagaaaaaagaacccataacgaaccgcgacattcacggtgcagacttgagt
        521  Q  R  L  E  K  K  E  P  I  T  N  R  D  I  H  G  A  D  L  S
RDS1:  1561 cagagattagagaaaaaagaacccataacgaaccgcgacattcacggtgcagacttgagt
        521  Q  R  L  E  K  K  E  P  I  T  N  R  D  I  H  G  A  D  L  S
ZZZ1:  1561 cagagattagagaaaaaagaacccataacgaaccgcgacattcacggtgcagacttgagt
        521  Q  R  L  E  K  K  E  P  I  T  N  R  D  I  H  G  A  D  L  S
LUP1:  1561 cagagattagagaaaaaagaacccataacgaaccgcgacattcacggtgcagacttgagt
        521  Q  R  L  E  K  K  E  P  I  T  N  R  D  I  H  G  A  D  L  S

BUL1:  1621 ggtaccattgatgattctattgaatcagactcccaagaaattttgcagaggaaattggac
        541  G  T  I  D  D  S  I  E  S  D  S  Q  E  I  L  Q  R  K  L  D
RDS1:  1621 ggtaccattgatgattctattgaatcagactcccaagaaattttgcagaggaaattggac
        541  G  T  I  D  D  S  I  E  S  D  S  Q  E  I  L  Q  R  K  L  D
ZZZ1:  1621 ggtaccattgatgattctattgaatcagactcccaagaaattttgcagaggaaattggac
        541  G  T  I  D  D  S  I  E  S  D  S  Q  E  I  L  Q  R  K  L  D
LUP1:  1621 ggtaccattgatgattctattgaatcagactcccaagaaattttgcagaggaaattggac
        541  G  T  I  D  D  S  I  E  S  D  S  Q  E  I  L  Q  R  K  L  D

BUL1:  1681 caactgcacattaagaacagaaataactatttagtcaactataacgatttgaagttgggc
        561  Q  L  H  I  K  N  R  N  N  Y  L  V  N  Y  N  D  L  K  L  G
RDS1:  1681 caactgcacattaagaacagaaataactatttagtcaactataacgatttgaagttgggc
        561  Q  L  H  I  K  N  R  N  N  Y  L  V  N  Y  N  D  L  K  L  G
ZZZ1:  1681 caactgcacattaagaacagaaataactatttagtcaactataacgatttgaagttgggc
        561  Q  L  H  I  K  N  R  N  N  Y  L  V  N  Y  N  D  L  K  L  G
LUP1:  1681 caactgcacattaagaacagaaataactatttagtcaactataacgatttgaagttgggc
        561  Q  L  H  I  K  N  R  N  N  Y  L  V  N  Y  N  D  L  K  L  G

BUL1:  1741 cacgatttggacaatggccgcagtggaaatagtggtcataataccgatacttccagagct
        581  H  D  L  D  N  G  R  S  G  N  S  G  H  N  T  D  T  S  R  A
RDS1:  1741 cacgatttggacaatggccgcagtggaaatagtggtcataataccgatacttccagagct
        581  H  D  L  D  N  G  R  S  G  N  S  G  H  N  T  D  T  S  R  A
ZZZ1:  1741 cacgatttggacaatggccgcagtggaaatagtggtcataataccgatacttccagagct
        581  H  D  L  D  N  G  R  S  G  N  S  G  H  N  T  D  T  S  R  A
LUP1:  1741 cacaatttggacaatggccgcagtggaaatagtggtcataataccgatacttccagagct
        581  H  N  L  D  N  G  R  S  G  N  S  G  H  N  T  D  T  S  R  A

BUL1:  1801 tggggtccctttgttgaaagtgaactaaaatataaactgaaaaacaaatccaattcctcc
        601  W  G  P  F  V  E  S  E  L  K  Y  K  L  K  N  K  S  N  S  S
RDS1:  1801 tggggtccctttgttgaaagtgaactaaaatataaactgaaaaacaaatccaattcctcc
        601  W  G  P  F  V  E  S  E  L  K  Y  K  L  K  N  K  S  N  S  S
ZZZ1:  1801 tggggtccctttgttgaaagtgaactaaaatataaactgaaaaacaaatccaattcctcc
        601  W  G  P  F  V  E  S  E  L  K  Y  K  L  K  N  K  S  N  S  S
LUP1:  1801 tggggtccctttgttgaaagtgaactaaaatataaactgaaaaacaaatccaattcctct
        601  W  G  P  F  V  E  S  E  L  K  Y  K  L  K  N  K  S  N  S  S
```

Figure 3.20: continued

```
BUL1:  1861 tcatttctgaacttctctcatttttaaacagcagttccagctcaatgtcctcttcctca
        621  S  F  L  N  F  S  H  F  L  N  S  S  S  S  S  M  S  S  S  S
RDS1:  1861 tcatttctgaacttctctcatttttaaacagcagttccagctcaatgtcctcttcctca
        621  S  F  L  N  F  S  H  F  L  N  S  S  S  S  S  M  S  S  S  S
ZZZ1:  1861 tcatttctgaacttctctcatttttaaacagcagttccagctcaatgtcctcttcctca
        621  S  F  L  N  F  S  H  F  L  N  S  S  S  S  S  M  S  S  S  S
LUP1:  1861 tcatttctgaacttctctcatttttaaacagcagttccagctcaatgtcctcttcctct
        621  S  F  L  N  F  S  H  F  L  N  S  S  S  S  S  M  S  S  S  S

BUL1:  1921 aatgcgggaaagaataatcatgatttaacgggaaataaagaaaggacagggctaatacta
        641  N  A  G  K  N  N  H  D  L  T  G  N  K  E  R  T  G  L  I  L
RDS1:  1921 aatgcgggaaagaataatcatgatttaacgggaaataaagaaaggacagggctaatacta
        641  N  A  G  K  N  N  H  D  L  T  G  N  K  E  R  T  G  L  I  L
ZZZ1:  1921 aatgcgggaaagaataatcatgatttaacgggaaataaagaaaggacagggctaatacta
        641  N  A  G  K  N  N  H  D  L  T  G  N  K  E  R  T  G  L  I  L
LUP1:  1921 aatgcgggaaagaataatcatgatttaacgggaaataaagaaaggacagggctaatacta
        641  N  A  G  K  N  N  H  D  L  T  G  N  K  E  R  T  G  L  I  L

BUL1:  1981 gtaaaggcgaaaattccaaaacagggcctaccatattgggctccctcattattgagaaag
        661  V  K  A  K  I  P  K  Q  G  L  P  Y  W  A  P  S  L  L  R  K
RDS1:  1981 gtaaaggcgaaaattccaaaacagggcctaccatattgggctccctcattattgagaaag
        661  V  K  A  K  I  P  K  Q  G  L  P  Y  W  A  P  S  L  L  R  K
ZZZ1:  1981 gtaaaggcgaaaattccaaaacagggcctaccatattgggctccctcattattgagaaag
        661  V  K  A  K  I  P  K  Q  G  L  P  Y  W  A  P  S  L  L  R  K
LUP1:  1981 gtaaaggcgaaaattccaaaacagggcctgccatattgggctccctcattattgagaaag
        661  V  K  A  K  I  P  K  Q  G  L  P  Y  W  A  P  S  L  L  R  K

BUL1:  2041 accaatgtttttgaatctaagagtaaacacgaccaagaaaattgggtgagattgtctgag
        681  T  N  V  F  E  S  K  S  K  H  D  Q  E  N  W  V  R  L  S  E
RDS1:  2041 accaatgtttttgaatctaagagtaaacacgaccaagaaaattgggtgagattgtctgag
        681  T  N  V  F  E  S  K  S  K  H  D  Q  E  N  W  V  R  L  S  E
ZZZ1:  2041 accaatgtttttgaatctaagagtaaacacgaccaagaaaattgggtgagattgtctgag
        681  T  N  V  F  E  S  K  S  K  H  D  Q  E  N  W  V  R  L  S  E
LUP1:  2041 accaatgtttttgaatctaagagtaaacatgaccaagaaaattgggtgagattgtctgag
        681  T  N  V  F  E  S  K  S  K  H  D  Q  E  N  W  V  R  L  S  E

BUL1:  2101 ttgattccggaagacgtaaaaaaaccattggaaaaacttgatttacaattgacttgcata
        701  L  I  P  E  D  V  K  K  P  L  E  K  L  D  L  Q  L  T  C  I
RDS1:  2101 ttgattccggaagacgtaaaaaaaccattggaaaaacttgatttacaattgacttgcata
        701  L  I  P  E  D  V  K  K  P  L  E  K  L  D  L  Q  L  T  C  I
ZZZ1:  2101 ttgattccggaagacgtaaaaaaaccattggaaaaacttgatttacaattgacttgcata
        701  L  I  P  E  D  V  K  K  P  L  E  K  L  D  L  Q  L  T  C  I
LUP1:  2101 ttgattccggaagacgtaaaaaaaccattggaaaaacttgatttacaattgacttgcata
        701  L  I  P  E  D  V  K  K  P  L  E  K  L  D  L  Q  L  T  C  I

BUL1:  2161 gaatccgataatagcttacctcatgatccgccagaaattcaatcgattaccacagaactg
        721  E  S  D  N  S  L  P  H  D  P  P  E  I  Q  S  I  T  T  E  L
RDS1:  2161 gaatccgataatagcttacctcatgatccgccagaaattcaatcgattaccacagaactg
        721  E  S  D  N  S  L  P  H  D  P  P  E  I  Q  S  I  T  T  E  L
ZZZ1:  2161 gaatccgataatagcttacctcatgatccgccagaaattcaatcgattaccacagaactg
        721  E  S  D  N  S  L  P  H  D  P  P  E  I  Q  S  I  T  T  E  L
LUP1:  2161 gaatccgataatagcttacctcatgatccgccagaaattcaatcgattaccacagaactg
        721  E  S  D  N  S  L  P  H  D  P  P  E  I  Q  S  I  T  T  E  L

BUL1:  2221 atatgtataactgctaaatctgataattctatcccaataaaactcaattctgaactgttg
        741  I  C  I  T  A  K  S  D  N  S  I  P  I  K  L  N  S  E  L  L
RDS1:  2221 atatgtataactgctaaatctgataattctatcccaataaaactcaattctgaactgttg
        741  I  C  I  T  A  K  S  D  N  S  I  P  I  K  L  N  S  E  L  L
```

Figure 3.20: continued

```
ZZZ1:  2221 atatgtataactgctaaatctgataattctatcccaataaaactcaattctgaactgttg
        741  I  C  I  T  A  K  S  D  N  S  I  P  I  K  L  N  S  E  L  L
LUP1:  2221 atatgtataacagctaaatctgataattctatcccaataaaactcaattctgaactgttg
        741  I  C  I  T  A  K  S  D  N  S  I  P  I  K  L  N  S  E  L  L

BUL1:  2281 atgaacaaagagaagctgacaagcatcaaagctttgtacgatgatttccattcaaaaatt
        761  M  N  K  E  K  L  T  S  I  K  A  L  Y  D  D  F  H  S  K  I
RDS1:  2281 atgaacaaagagaagctgacaagcatcaaagctttgtacgatgatttccattcaaaaatt
        761  M  N  K  E  K  L  T  S  I  K  A  L  Y  D  D  F  H  S  K  I
ZZZ1:  2281 atgaacaaagagaagctgacaagcatcaaagctttgtacgatgatttccattcaaaaatt
        761  M  N  K  E  K  L  T  S  I  K  A  L  Y  D  D  F  H  S  K  I
LUP1:  2281 atgaacaaagaaaagctgacaagcatcaaagctttgtacgatgatttccattcaaaaatt
        761  M  N  K  E  K  L  T  S  I  K  A  L  Y  D  D  F  H  S  K  I

BUL1:  2341 tgtgaatatgaaaccaagttcaacaagaattttcttgaattaaatgagttatataatatg
        781  C  E  Y  E  T  K  F  N  K  N  F  L  E  L  N  E  L  Y  N  M
RDS1:  2341 tgtgaatatgaaaccaagttcaacaagaattttcttgaattaaatgagttatataatatg
        781  C  E  Y  E  T  K  F  N  K  N  F  L  E  L  N  E  L  Y  N  M
ZZZ1:  2341 tgtgaatatgaaaccaagttcaacaagaattttcttgaattaaatgagttatataatatg
        781  C  E  Y  E  T  K  F  N  K  N  F  L  E  L  N  E  L  Y  N  M
LUP1:  2341 tgtgaatatgaaaccaagttcaacaagaatttccttgaattaaatgagttatataatatg
        781  C  E  Y  E  T  K  F  N  K  N  F  L  E  L  N  E  L  Y  N  M

BUL1:  2401 aataggggagaccgtaggccaaaggaactgaaatttacagattttattacttcacagctg
        801  N  R  G  D  R  R  P  K  E  L  K  F  T  D  F  I  T  S  Q  L
RDS1:  2401 aataggggagaccgtaggccaaaggaactgaaatttacagattttattacttcacagctg
        801  N  R  G  D  R  R  P  K  E  L  K  F  T  D  F  I  T  S  Q  L
ZZZ1:  2401 aataggggagaccgtaggccaaaggaactgaaatttacagattttattacttcacagctg
        801  N  R  G  D  R  R  P  K  E  L  K  F  T  D  F  I  T  S  Q  L
LUP1:  2401 aataggggagaccgtaggccaaaggaactgaaatttacagattttattaccctcacagctg
        801  N  R  G  D  R  R  P  K  E  L  K  F  T  D  F  I  T  S  Q  L

BUL1:  2461 tttaacgatatcgaaagcatttgcaacttgaaagttagtgttcacaacttatccaacatt
        821  F  N  D  I  E  S  I  C  N  L  K  V  S  V  H  N  L  S  N  I
RDS1:  2461 tttaacgatatcgaaagcatttgcaacttgaaagttagtgttcacaacttatccaacatt
        821  F  N  D  I  E  S  I  C  N  L  K  V  S  V  H  N  L  S  N  I
ZZZ1:  2461 tttaacgatatcgaaagcatttgcaacttgaaagttagtgttcacaacttatccaacatt
        821  F  N  D  I  E  S  I  C  N  L  K  V  S  V  H  N  L  S  N  I
LUP1:  2461 tttaacgatatcgaaagcatttgcaacttgaaagttagtgttcacaacttatccaacatt
        821  F  N  D  I  E  S  I  C  N  L  K  V  S  V  H  N  L  S  N  I

BUL1:  2521 tttaaaaaacaggtcagtaccctaaaacaacactcaaagcacgcattatctgaggattca
        841  F  K  K  Q  V  S  T  L  K  Q  H  S  K  H  A  L  S  E  D  S
RDS1:  2521 tttaaaaaacaggtcagtaccctaaaacaacactcaaagcacgcattatctgaggattca
        841  F  K  K  Q  V  S  T  L  K  Q  H  S  K  H  A  L  S  E  D  S
ZZZ1:  2521 tttaaaaaacaggtcagtaccctaaaacaacactcaaagcacgcattatctgaggattca
        841  F  K  K  Q  V  S  T  L  K  Q  H  S  K  H  A  L  S  E  D  S
LUP1:  2521 tttaaaaaacaggtcagtaccctaaaacaacactcaaagcacgcattatctgaggattca
        841  F  K  K  Q  V  S  T  L  K  Q  H  S  K  H  A  L  S  E  D  S

BUL1:  2581 atatcgcacacaggtaacggtagttcatcgtcgcccagttcagcgtcattaacgccagta
        861  I  S  H  T  G  N  G  S  S  S  S  P  S  S  A  S  L  T  P  V
RDS1:  2581 atatcgcacacaggtaacggtagttcatcgtcgcccagttcagcgtcattaacgccagta
        861  I  S  H  T  G  N  G  S  S  S  S  P  S  S  A  S  L  T  P  V
ZZZ1:  2581 atatcgcacacaggtaacggtagttcatcgtcgcccagttcagcgtcattaacgccagta
        861  I  S  H  T  G  N  G  S  S  S  S  P  S  S  A  S  L  T  P  V
LUP1:  2581 atatcgcacacaggtaacggtaattcatcgtcgcccagttcagcgccattaacgccagta
        861  I  S  H  T  G  N  G  N  S  S  S  P  S  S  A  P  L  T  P  V
```

Figure 3.20: continued

```
BUL1:  2641 acttcttcatccaagagtagtttatttttacctagcggtagctcgtctacttccctgaaa
        881  T  S  S  S  K  S  S  L  F  L  P  S  G  S  S  S  T  S  L  K
RDS1:  2641 acttcttcatccaagagtagtttatttttacctagcggtagctcgtctacttccctgaaa
        881  T  S  S  S  K  S  S  L  F  L  P  S  G  S  S  S  T  S  L  K
ZZZ1:  2641 acttcttcatccaagagtagtttatttttacctagcggtagctcgtctacttccctgaaa
        881  T  S  S  S  K  S  S  L  F  L  P  S  G  S  S  S  T  S  L  K
LUP1:  2641 acttcttcatccaagagtagtttatttttacctagcagtagctcgtctacttccctgaag
        881  T  S  S  S  K  S  S  L  F  L  P  S  S  S  S  S  T  S  L  K

BUL1:  2701 tttacagaccagattgttcataaatgggttaggattgctcctttacagtacaaacgagac
        901  F  T  D  Q  I  V  H  K  W  V  R  I  A  P  L  Q  Y  K  R  D
RDS1:  2701 tttacagaccagattgttcataaatgggttaggattgctcctttacagtacaaacgagac
        901  F  T  D  Q  I  V  H  K  W  V  R  I  A  P  L  Q  Y  K  R  D
ZZZ1:  2701 tttacagaccagattgttcataaatgggttaggattgctcctttacagtacaaacgagac
        901  F  T  D  Q  I  V  H  K  W  V  R  I  A  P  L  Q  Y  K  R  D
LUP1:  2701 tttacagaccagattgttcataaatgggttaggattgctcctttacagtacaaacgagac
        901  F  T  D  Q  I  V  H  K  W  V  R  I  A  P  L  Q  Y  K  R  D

BUL1:  2761 attaatgtgaacttggaatttaataaggacattaaggaaactttaattccaagttttgaa
        921  I  N  V  N  L  E  F  N  K  D  I  K  E  T  L  I  P  S  F  E
RDS1:  2761 attaatgtgaacttggaatttaataaggacattaaggaaactttaattccaagttttgaa
        921  I  N  V  N  L  E  F  N  K  D  I  K  E  T  L  I  P  S  F  E
ZZZ1:  2761 attaatgtgaacttggaatttaataaggacattaaggaaactttaattccaagttttgaa
        921  I  N  V  N  L  E  F  N  K  D  I  K  E  T  L  I  P  S  F  E
LUP1:  2761 attaatgtgaacttggaatttaataaggacattaaggaaactttaattccaagttttgaa
        921  I  N  V  N  L  E  F  N  K  D  I  K  E  T  L  I  P  S  F  E

BUL1:  2821 agctgcctatgttgtaggttttattgcgttcgagtaatgattaaatttgaaaaccatctt
        941  S  C  L  C  C  R  F  Y  C  V  R  V  M  I  K  F  E  N  H  L
RDS1:  2821 agctgcctatgttgtaggttttattgcgttcgagtaatgattaaatttgaaaaccatctt
        941  S  C  L  C  C  R  F  Y  C  V  R  V  M  I  K  F  E  N  H  L
ZZZ1:  2821 agctgcctatgttgtaggttttattgcgttcgagtaatgattaaatttgaaaaccatctt
        941  S  C  L  C  C  R  F  Y  C  V  R  V  M  I  K  F  E  N  H  L
LUP1:  2821 agctgcttatgttgtaggttttattgcgttcgagtaatgattaaatttgaaaaccatctt
        941  S  C  L  C  C  R  F  Y  C  V  R  V  M  I  K  F  E  N  H  L

BUL1:  2881 ggcgtagcgaagattgatatccctatttctgttaggcaagtgacaaaataaaaaaacatt
        961  G  V  A  K  I  D  I  P  I  S  V  R  Q  V  T  K  976
RDS1:  2881 ggcgtacggaagattgatatccctatttctgttaggcaagtgacaaaataaaaaaacatt
        961  G  V  R  K  I  D  I  P  I  S  V  R  Q  V  T  K  976
ZZZ1:  2881 ggcgtagcgaagattgatatccctatttctgttaggcaagtgacaaaataaaaaaacatt
        961  G  V  A  K  I  D  I  P  I  S  V  R  Q  V  T  K  976
LUP1:  2881 ggcgtagcgaagattgatatccctatttctgttaggcaagtgacaaaataaaaaaacatt
        961  G  V  A  K  I  D  I  P  I  S  V  R  Q  V  T  K  976
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4. DISCUSSION

4.1 LUP$^+$ STRAINS ARE SENSITIVE TO HYDROPHOBIC AND NONHYDROPHOBIC TOXIC AMINO ACID ANALOGS

Yeast with only the *lup1* allele accumulate hydrophobic amino acids better than *LUP1* strains, in the presence of ammonium (Heinemann et al., 1994). Lup$^+$ variants would therefore be predicted to be more sensitive than their progenitor to the toxic analogs of these hydrophobic amino acids. Results of PGAs (Table 3.1) and MIC determinations (Table 3.2), carried out in the presence of ammonium, suggested that 329-6C (*LUP1*) and JY127 (*LUP1/lup1*) were not only more resistant, as expected, to the hydrophobic amino acid analogs *m*-fluoro-D,L-phenylalanine, L-methionine sulphoximine and L-ethionine, but unexpectedly, the nonhydrophobic amino acid analogs L-azaserine and canavanine. These latter results were unexpected because [^{14}C]leucine transport inhibition studies carried out by Heinemann et al. (1994) found a correlation between amino acid competitor hydrophobicity and leucine uptake inhibition. Amino acids with transfer free energies below 1.9 kcal mol^{-1} did not inhibit leucine uptake by Lup$^+$ cells. However, we found azaserine and canavanine, analogs of the very hydrophilic amino acids glutamine and arginine, respectively, were differentially more toxic to Lup$^+$ cells, suggesting increased uptake of these compounds by the putative Lup permease. The results of this study may indicate that the Lup permease recognises more substrate characteristics than just hydrophobicity. Additional characteristics may include chain length in conjunction with minimum hydrogen bonding capability. Whether the mechanism of increased sensitivity of *lup1* cells to L-canavanine and L-azaserine is in fact due to increased uptake of these compounds remains to be determined. A comparison of uptake of [^{14}C]-labelled L-canavanine or arginine and L-azaserine or glutamine by *lup1* and *LUP1* strains should resolve this issue.

As all amino acid uptake permeases studied in yeast thus far are structurally similar, an alternative explanation for the results obtained could be that Lup1 may regulate additional amino acid permeases, such as those that import nonhydrophobic amino acids. Heinemann et al. (1994) determined the amino acid specificity of the Lup

permease by ascertaining percentage inhibition of leucine transport in the Lup^+ strain by competing amino acids. If Lup1 was also regulating a hydrophilic amino acid permease, amino acids transported by this permease would not inhibit leucine uptake by the separate, Lup pathway. *NPI1/RSP5*, *NPR1*, *URE2*, *DAL5*, *SSY1* and *GLN3* exemplify only a few of numerous examples of genes encoding amino acid permease regulators which, when mutated, influence the function of multiple permeases. Therefore, that Lup1 may regulate greater than one amino acid permease would not be a surprise.

4.2 TWO GENES CONFER FPA^R

The clones that complemented the Fpa^S phenotype of Lup^+ strains differed with respect to the Lup phenotype conferred, thus two separate genes had been isolated with Fpa^S-complementing ability. Restriction and Southern analyses confirmed that the Fpa^R, Lup^--conferring clones were distinct from those that only conferred Fpa^R. The gene that confers the Fpa^R, Lup^- phenotypes was identified as *BUL1* (Yashiroda et al., 1996) by sequence similarity. The gene that complements the Fpa^S, but not Lup^+, phenotype was identified by similar methods as *ARO4* (Künzler et al., 1992).

It was considered unlikely that *ARO4* and *LUP1* were the same alleles, as *BUL1* complemented both phenotypes characteristic of the *lup1* mutant whereas *ARO4* only complemented one. The 2.04 kb *ARO4* gene encodes the tyrosine-regulated 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase which catalyses the first step in the Shikimate pathway for aromatic amino acid biosynthesis (Künzler et al., 1992). Fukuda et al. (1992) have shown that a single nucleotide change in *ARO4*, resulting in substitution of glutamine by lysine in the structural region of DAHP synthase, causes feedback insensitivity and resistance to *o*-fluoro-D,L-phenylalanine. Moreover, *aro4* mutants are available as a dominant selection marker for fluorophenylalanine resistance in yeast vectors. *ARO4* clones found to complement the Fpa^S phenotype of *lup1* strains were only isolated from the Botstein library, carried by the high-copy number vector YEp24 (25-200 copies per cell). Perhaps *ARO4* conferred Fpa^R when overexpressed, or, alternatively, the Botstein library may have been created from a strain containing *aro4*. Both yeast and *E. coli* possess a phenylalanine-sensitive form of DAHP synthase

in addition to the tyrosine-repressible form. In *E. coli*, the phenylalanine-sensitive DAHP synthase is inhibited by phenylalanine analogs such as fluorophenylalanine, as is the tyrosine-regulated form when analogs are added at higher concentrations (Fowden et al., 1967). That overexpression of *ARO4* or a mutation in this gene confers Fpa^R suggests that the yeast tyrosine-regulated DAHP synthase is also regulated to some extent by phenylalanine.

4.3 *LUP1* IS ALLELIC TO *BUL1*

4.3.1 *LUP1* and *BUL2* are not Allelic

Although *BUL1* complemented the Fpa^S and Lup^+ phenotypes of *lup1* variants, it is premature to conclude that *LUP1* is allelic to *BUL1*. It is possible that more than one gene exists that can complement these phenotypes. Indeed, we have seen that the product of *ARO4* is capable of complementing the Fpa^S phenotype of *lup1* mutants. Moreover, a gene, *BUL2*, has recently been described that encodes a protein with similar structure (51% identity) and function to Bul1 (Yashiroda et al., 1998). Therefore, *BUL2* might also complement the Lup^- and Fpa^S phenotypes.

The possibility that *BUL2* and *LUP1* are alleles of the same gene was excluded, however. Deletion of *BUL2* from the chromosome did not result in a strain with increased sensitivity to *m*-fluoro-D,L-phenylalanine or an increased leucine-scavenging ability. Furthermore, expression of *BUL2* from a single copy plasmid was incapable of complementing the Fpa^S and Lup^+ phenotypes of a *lup1* mutant.

4.3.2 *bul1* and *lup1* Confer Identical Phenotypes

If a *bul1* mutant displays the same phenotypes as a *lup1* mutant, this would provide evidence that *BUL1* and *LUP1* are allelic. We therefore replaced the chromosomal *BUL1* locus with a *bul1::URA3* allele. Consistent with *BUL1* and *LUP1* being allelic, we found that all strains which had replaced *BUL1* with the *bul1::URA3* became Fpa^S and Lup^+. Moreover, whilst *bul1* and *BUL1* strains grow normally at 30°C, *bul1*

variants differ from their wildtype counterparts by an inability to grow at 37°C (Yashiroda et al. 1996). A comparison of the abilities of *LUP1*, *lup1* and *bul1::URA3* strains to grow at 30°C and 37°C has revealed that like *bul1* mutants, growth of *lup1* variants was also temperature sensitive.

4.3.2.1 Where has Integration Occurred In Lup⁻ Transformants?

When the 'knockout' *BUL1* allele was being constructed, approximately 10% of variants that integrated the *bul1::URA3* allele had not replaced *BUL1*. Chromosomal integration of linear DNA fragments within yeast occurs at chromosomal sequences homologous to the linear fragment termini (Orr-Weaver et al., 1981). Therefore, as the linear ends of the *bul1::URA3* construct consisted of *BUL1* sequence and sequence downstream of *BUL1*, we predicted that all replacement events would occur at the *BUL1* locus. We were therefore interested in where the linear fragment had integrated and why integration did not always occur at the *BUL1* locus.

Insertion of the linear knockout construct may have occurred downstream of *BUL1* rather than partially replacing this allele, resulting in a partial duplication of the *BUL1* gene. No partial duplications were detected by restriction and Southern analyses of PCR products of *BUL1* and surrounding regions from integrants remaining Lup⁻, however. Alternatively, the linear knockout construct may have integrated at another site with high sequence similarity to one of its termini. The first 79 nucleotides of the construct termini with approximately 100 nucleotides of *BUL1* sequence, had 73% identity to the *BUL2* sequence. Restriction analyses of *BUL2*, amplified by PCR from Lup$^+$ and Lup⁻ integrants, have likewise confirmed that integration did not occur there. Integration may have occurred at the *URA3-52* locus, although this would be surprising. Mutation at the *URA3-52* locus is created by Ty insertion, which almost completely eliminates *URA3* replacement events, and integration events are reduced, although not eliminated (Rose and Winston, 1984).

It may not be surprising that integration did not always occur at *BUL1*, as it is not known exactly how much identity is required between linear fragment termini and chromosomal DNA for efficient targeting. Wach et al. (1994) have reported that 95% of integration events consisted of replacement of the target gene when only 30 to 45

nucleotides of target DNA flanked the marker gene in the linear knockout construct. However, Rose and Broach (1991) suggested that termini containing less than 250 nucleotides of target sequence are not targeted easily, whereas greater than 500 nucleotides are considered sufficient.

4.3.3 *LUP1* and *BUL1* Occupy the Same Chromosomal Position

Proof that *BUL1* and *LUP1* are alleles of the same gene requires a genetic test of location. The phenotypes of a diploid created from a cross between a *bul1* and a *lup1* mutant, and the offspring following sporulation, were studied to assess whether *BUL1* and *LUP1* lie in the same chromosomal position. If the haploid phenotypes are complemented in the diploid, resulting in the wildtype phenotype, then the mutations most likely lie within different genes. If, however, the diploid has the mutant phenotype, then mutations are probably occurring within the same gene. Therefore, assuming that *LUP1* and *BUL1* are the same allele (Figure 3.17, Scenario 2), we predicted that the diploid created from a cross between a *bul1* and a *lup1* mutant would display Lup^+ and Fpa^S phenotypes. We were unable to predict the diploid phenotypes, assuming *LUP1* and *BUL1* are separate alleles (Figure 3.17, Scenario 1), however. Phenotypes of *lup1* and *bul1* mutants are recessive to *LUP1* and *BUL1* variants, respectively, thus the diploid may be expected to exhibit Lup^- and Fpa^R phenotypes. *BUL1* complemented phenotypes of *lup1* strains when carried by a plasmid, although spontaneously arising *lup1* mutants in a *BUL1* strain had Lup^+ and Fpa^S phenotypes. This suggested that if *LUP1* and *BUL1* are separate alleles, phenotypes due to the *lup1* allele are only dominant over those conferred by *BUL1* when a single copy of *BUL1* is present. Conversely, disruption of *BUL1* in a *LUP1* strain also resulted in the Lup^+ phenotype. The phenotypes of the diploid created in this study were Lup^+ and Fpa^S, supporting allelism between *LUP1* and *BUL1*, but not eliminating the possibility that the *BUL1* and *LUP1* may be separate genes.

Assuming *LUP1* and *BUL1* are separate, unlinked alleles, haploid offspring resulting from sporulation of a *LUP1 lup1*, *BUL1 bul1* diploid would have a 1:1:1:1 ratio of the genotypes *lup1 bul1*:*lup1 BUL1*:*LUP1 bul1*:*LUP1 BUL1*, equating to a 3:1 ratio of Lup^+:Lup^- phenotypes. The more closely linked the alleles are, the less frequently the

LUP1 BUL1 genotype and wildtype phenotype will arise. Thus, if both mutations occur in the identical position within the same gene, 100% of offspring will portray the mutant phenotype (unless spontaneous reversion occurs). All 1083 progeny, assayed following diploid sporulation, exhibited the mutant Fpa^S and Lup^+ phenotypes. These results indicate that the mutations within *lup1* and *bul1* occur at an extremely close proximity on the chromosome, within a distance of less than 0.18 cM.

4.3.4 Lup^+ Variants have a Different *BUL1* Sequence than Wildtype

Assuming *LUP1* is allelic to *BUL1*, we predicted that Lup^+ mutants would contain a different *BUL1* DNA sequence from wildtype. Indeed, the *BUL1* sequence from two spontaneously and independently arising Lup^+ variants each differed be one base from the *BUL1* sequence of the wildtype background from which they were derived. For strain JY117, deletion of base number 2010 of the *BUL1* coding sequence resulted in a frameshift mutation. Lup^+ mutant JOY53 had undergone a transversion mutation at *BUL1* nucleotide number 1008, resulting in the replacement of cytosine (C) by adenine (A), and corresponding to an amino acid change from serine (S) to arginine (R).

4.4 REGULATION OF LUP PERMEASE BY LUP1/BUL1

LUP1/BUL1, a 3 kb gene with a predicted gene product that appears to be a basic and hydrophillic 110 kDa protein (Yashiroda et al., 1996), has been independently isolated by a number of researchers. Biggins et al. (1996) sequenced *LUP1/BUL1* while sequencing an adjacent gene, *DSK2*, and named it *DAG1* (*DSK2* Adjacent Gene). The gene has also been entered on the SWISS-PROT database under the name *RDS1*, for Respiration Deficiency Suppressor, however, to our knowledge, no reference is available describing this property. Yashiroda et al. (1996) have described a temperature sensitive *bul1* mutant in which, unlike a wildtype *BUL1* strain, a YCp50 minichromosome was maintained less stably at 35°C (a semipermissive temperature) than at 26°C (a permissive temperature). Temperature sensitivity of *bul1* mutants could be suppressed by adding 1 M sorbitol to the media as an osmotic stabiliser. Disruption of *BUL1* resulted in sensitivity to a number of stresses in addition to temperature

sensitivity, including high salt concentrations such as 1.2 M sodium chloride, the non-fermentable carbon source glycerol, and 100 mM lithium chloride. Stress sensitivity of *bul1* mutants was strain-dependent, although strains with double *BUL1* and *BUL2* disruptions were sensitive regardless of strain background (Yashiroda et al., 1998). Fisk and Yaffe (1999) have isolated *lup1/bul1* mutants by their partial ability to suppress temperature sensitive growth and defects in mitochondrial inheritance and morphology resulting from the *mdm1-252* mutation. As *lup1/bul1* strains were not defective in mitochondrial inheritance, Lup1/Bul1 may not be normally involved in this process. Fisk and Yaffe (1999) have suggested that a similar protein, such as Bul2, may be involved instead, and mutations within *LUP1/BUL1* may allow Lup1/Bul1 to interfere with or supplement Bul2 activity. Finally, Wolfe et al. (1999) have isolated mutants that are resistant to the growth-inhibitory effects of a number of volatile anaesthetics. Seven mutants contained mutations in the *ZZZ1* gene, which was shown to be identical to *LUP1/BUL1*. Thus Lup1/Bul1 appears to be involved in the yeast response to anaesthetics. In addition, *zzz1* mutants exhibited greater cadmium sensitivity than their wildtype counterparts.

Several lines of evidence indicate that Lup1/Bul1 and the ubiquitin ligase, Rsp5, interact. Using the yeast two-hybrid system, Lup1/Bul1 was found to bind Rsp5, and *BUL1* was named regarding this feature (Binds to Ubiquitin Ligase). Moreover, Lup1/Bul1 and Rsp5 cosedimented through sucrose density gradient centrifugation and coimmunoprecipitated. Interaction between Lup1/Bul1 and Rsp5 raised two possibilities: (1) Lup1/Bul1 is a substrate for Rsp5-mediated degradation via the ubiquitin pathway, or (2) Rsp5 activity is modulated via Lup1/Bul1. The former possibility predicts that Lup1/Bul1 stability would be increased in a *rsp5* background. Lup1/Bul1 was found to be a stable protein, however, and stability was not increased by mutation of *RSP5*. Assuming Rsp5 activity may be modulated though Lup1/Bul1, mutations in *LUP1/BUL1* should result in similar phenotypes to mutations in *RSP5*. Indeed, many phenotypes displayed by *rsp5* and *lup1/bul1* mutants were identical. The *rsp5-101* allele was viable, and like *lup1/bul1* mutants, *rsp5-101* strains were temperature sensitive (Yashiroda et al., 1996). This study has demonstrated that *rsp5-101* mutants exhibit the same Fpa^S, but not necessarily Lup^+, phenotypes as *lup1* variants. *rsp5-101* strains were also not defective in minichromosome stability (Yashiroda et al., 1996) or salt-sensitive (Yashiroda et al., 1998). Like *lup1/bul1*

disruptants, strains containing the mutated *RSP5* alleles *mdp1-1* and *mdp1-16* were resistant to the growth inhibitory effects of the anaesthetic isoflurane (Wolfe et al., 1999).

It is therefore possible that Rsp5 function is modulated via Lup1/Bul1, and further experiments have indicated that interaction with Rsp5 may be essential for Lup1/Bul1 function. Rsp5 contains three WW domains, which are protein-protein interaction modules with an affinity for proline-rich sequences with the consensus binding sequence PPxY (PY-motif). WW domains are required for substrate-binding by Rsp5 and thus, for Rsp5 function (Wang et al., 1999). Alteration of the PY-motif present at the N-terminal region of Lup1/Bul1, from PPSY to QASY, eliminated the ability of Lup1/Bul1 to bind Rsp5 in the two-hybrid system, and virtually abolished coprecipitation with Rsp5. Moreover, the *lup1/bul1* allele encoding the altered PY-motif (*bul1*$^{P157Q,\ P158A}$) was unable to overcome temperature or salt sensitivity or inability to utilise glycerol, caused by *LUP1/BUL1* disruption (Yashiroda et al., 1998). Similarly, we have seen a failure of the *bul1*$^{P157Q,\ P158A}$ allele to complement the Fpa^{S} and Lup^{+} phenotypes of a *lup1* mutant, when expressed from a single copy plasmid. Lup1/Bul1 function therefore requires the PY-motif, probably for binding to Rsp5, although we cannot rule out the possibility that changes within this motif might affect the structure of the whole protein.

In addition to its association with Rsp5, further evidence also supports the involvement of Lup1/Bul1 in the ubiquitin pathway. First, a high dose of *UBI1*, encoding ubiquitin, partially suppressed the temperature sensitivity of *lup1/bul1* strains (Yashiroda et al., 1996). Also, protein degradation occurs in the proteasome or vacuole following ubiquitination in yeast, and Wolfe et al. (1999) have demonstrated the function of the proteasome in the Lup1/Bul1-dependent cellular anaesthetic response. Moreover, disruption of the ubiquitin pathway E4 enzyme, Zzz4/Doa1, resulted in a similarly altered response to anaesthetics as Lup1/Bul1 disruption (Wolfe et al., 1999). If Lup1/Bul1 and Zzz4/Doa1 are components of separate pathways involved in the anaesthetic response, an additive effect would be predicted if these proteins were simultaneously disrupted. The anaesthetic response of the *zzz1 zzz4/doa1* double mutant was found to be no greater than that of a *zzz1* or *zzz4/doa1* single mutant, however. Evidence therefore implicates the involvement of Bul1 in the ubiquitin

pathway, and Yashiroda et al. (1996) have proposed that a Lup1/Bul1-Rsp5 complex functions as an E3 component involved in substrate recognition for protein ubiquitination. A similar E3 role is played by a complex of the human ubiquitin protein ligase E6-AP with the E6 protein of human papilloma virus in the ubiquitination and subsequent proteolysis of p53.

A number of diverse yeast plasma membrane proteins have been found to be regulated by ubiquitination (Table 4.1) which involves, in at least four of these examples, the function of the ubiquitin ligase Rsp5. Interestingly, one such protein is the general amino acid permease, Gap1. Rsp5-dependent, ubiquitin-directed degradation of Gap1 is triggered by the presence of ammonium in the environment. We propose an analogous NCI-type mechanism for the negative regulation of the Lup permease in the presence of ammonium, involving a Lup1/Bul1-Rsp5 E3-type complex for substrate recognition (Figure 4.1). In the presence of ammonium, the Lup permease is recognised and ubiquitinated by the Lup1/Bul1-Rsp5 complex, and subsequently undergoes endocytosis and degradation in the vacuole, causing cessation of hydrophobic amino acid transport via the permease. In the presence of proline as a nitrogen source, or in *lup1/bul1* strains grown in either nitrogen source, the permease is not ubiquitinated and hence, not degraded. Thus amino acid transport through the Lup permease is allowed to continue.

Table 4.1: Ubiquitinated plasma membrane proteins in *S. cerevisiae*.

Membrane Protein	Rsp5 Involvement	Reference
ABC peptide transporter (Ste6)	Not determined	Kölling and Hollenberg, 1995
a-factor receptor (Ste3)	Not determined	Roth and Davis, 1996
α-factor receptor (Ste2)	Not determined	Hicke and Riezman, 1996
Uracil permease (Fur4)	Yes	Galan et al., 1996
Multidrug transporter (Sts1)	Not determined	Egner and Kuchler, 1996
Galactose permease (Gal2)	Not determined	Horak and Wolf, 1997
Maltose permease (Mal61)	Yes	Riballo et al., 1995
Zinc permease (Zrt1)	Yes	Gitan and Eide, 2000
General amino acid permease (Gap1)	Yes	Hein et al., 1995

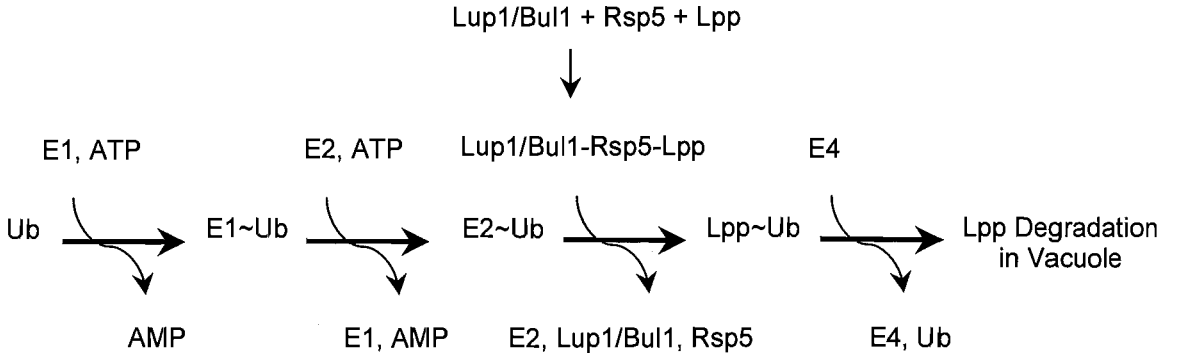

Figure 4.1: Proposed ubiquitin-dependent mechanism for regulation of Lup Permease (Lpp) involving Lup1/Bul1, in the presence of ammonium. In an ATP-dependent reaction, ubiquitin (Ub) is activated by an ubiquitin-activating enzyme (E1), forming a high-energy, thiolester (E1~Ub). Activated ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2), forming an E2-ubiquitin thiolester (E2~Ub). Finally, ubiquitin is transferred to the substrate, Lpp, in a reaction requiring the Lup1/Bul1-Rsp5 complex for substrate recognition. Once ubiquitinated, Lpp is targeted for degradation, and ubiquitin is recycled by one or more E4 enzymes.

How does the nitrogen source influence Lup1/Bul1 regulation of the Lup permease? Lup1/Bul1 may be regulated by the nitrogen source. If so, Lup1/Bul1 would be expected to be positively regulated or derepressed by ammonium, and negatively regulated or constitutive in the presence of proline. In yeast, NCR is the only mechanism identified thus far that regulates transcription in response to the quality of the nitrogen source present. Transcription of NCR-sensitive genes is negatively regulated in the presence of readily utilisable nitrogen sources and positively regulated when poor nitrogen sources are available, indeed the opposite to how we would predict that *LUP1/BUL1* would be regulated in response to nitrogen source. Also, *LUP1/BUL1* transcription was not identified as nitrogen-regulated in a screen for NCR-sensitive genes (Cox et al., 1999). These researchers compared transcripts from wildtype, and *gln3* and *dal80* mutants from repressive and nonrepressive nitrogen environments, using genome-wide transcriptional analysis by mini-array membrane hybridisation. The screen accurately identified genes known to be regulated by NCR, and detected a number of genes not previously reported to be controlled by the nitrogen source.

Lup1/Bul1 may be regulated post-translationally in response to the nitrogen source. It is possible that phosphorylation controls Lup1/Bul1 function. Lup1/Bul1 has many potential phosphorylation sites including those that are recognised by mitogen-activated protein (MAP) kinase, CDC28 kinase, protein kinase A (PKA), C kinase and casein kinase II, and preliminary results reported by Yashiroda et al. (1996) indicated that Lup1/Bul1 may indeed be phosphorylated. Moreover, the kinases that possibly phosphorylate Lup1/Bul1 are involved in responses and environmental changes that Lup1/Bul1 is also associated with. MAP kinase, PKA and C protein kinases are integral members of signal transduction pathways that mediate cellular processes such as cell growth and adaptation to stress, in response to a range of environmental stresses such as nutrient limitation, heat shock (Boy-Marcotte et al., 1999), osmotic, ionic and oxidative stresses (Garay-Arroyo and Covarrubias, 1999; Nguyen and Shiozaki, 1999). Evidence presented by Lorenz and Heitman (1998a) suggests that the putative ammonium sensor, Mep2, may serve to regulate pseudohyphal growth in response to ammonium levels by functioning in a signalling pathway upstream of the RAS/PKA pathway. Also, the involvement of this pathway has been implicated in the regulation of leucine transport through Bap2 and S2 permeases in response to nitrogen source of the medium (Sáenz et al., 1997). Moreover, a leucine permease in *S. pombe* is down-regulated in response to ammonium by Pub1, an E3 ubiquitin ligase similar to Rsp5 (Karagiannis et al., 1999). Ammonium repression was abrogated by disruption of *MSC4*, which encodes an upstream regulator of the MAP kinase pathway. An interaction between MAP kinase and ubiquitin pathways has been reported for other systems, including in human B and T cells where phosphorylation of the bcl-6 proto-oncogene by the MAP kinase targets its destruction via the ubiquitin pathway (Niu et al., 1998).

It is possible that Lup1/Bul1 function may not be controlled at all by the nitrogen source. Pleiotropic effects of *LUP1/BUL1* disruption indicate that Lup1/Bul1 is required for functions in addition to regulation of the Lup permease in response to nitrogen source. Lup1/Bul1 function would therefore be required in all nitrogen environments. If Lup1/Bul1 was controlled at the level of protein production or turnover in response to the nitrogen source, Lup1/Bul1 would not be available for its other functions in nitrogen-limiting conditions. The decision whether Gap1 undergoes Rsp5-mediated ubiquitination and subsequent degradation is thought to be determined by Gap1 phosphorylation by Npr1. Phosphorylation of Gap1 in conditions of poor

nitrogen source protects the permease against ubiquitination. In the presence of ammonium, mutations in *NPR1* repress the activity of at least four additional ammonium-sensitive uptake permeases, Put4, Dal5, Mep1 and Mep2 (Grenson and Dubois, 1982), and derepress Tat2 (Schmidt et al., 1998). The ubiquitous nature of permease regulation in response to nitrogen source by Npr1 raises the possibility that phosphorylation of the Lup permease by this factor may also be responsible for determining whether this permease is recognised and ubiquitinated by the Lup1/Bul1-Rsp5 complex.

Additional components that are involved in the Lup1/Bul1-Rsp5 complex-dependent regulation of the Lup permease remain to be identified. The E2 enzymes Ubc4 and Ubc5 are important for degradation of short-lived and abnormal proteins and are required to enable the cell to withstand stresses such as heat shock and toxic amino acid analog exposure (Seufert and Jentsch, 1990). Nuber and Scheffner (1999) have identified that Ubc4 and Ubc5 interact with the hect domain of Rsp5 and have identified the region within Ubc4 and Ubc5 responsible for this interaction. Moreover, these E2 proteins are required for Rsp5-mediated activation of the plasma membrane proton-ATPase (de la Fuente et al., 1997) and ubiquitination of the maltose permease (Medintz et al., 1998). These proteins may therefore also function in Lup permease regulation. Disruptions of the E4 enzyme, Doa4/Npi2, result in strong inhibition of ubiquitination, internalization and degradation of the Rsp5-regulated maltose (Lucero and Lagunas, 1997; Medintz et al., 1998), uracil (Galan and Haguenauer-Tsapis, 1997) and Gap1 permeases (Springael et al., 1999b). Also, another E4 enzyme, Doa1/Zzz4, is involved in the Lup1/Bul1-dependent anaesthetic response (Wolfe et al., 1999). Whether either of these enzymes is involved in the Lup1/Bul1-dependent down regulation of the Lup permease in the presence of ammonium remains to be determined. Indeed, both enzymes may be involved directly, as distinct ubiquitin-hydrolases serve different functions at different stages of proteolysis, or indirectly, by recycling ubiquitin and maintaining the general ubiquitin pool.

The model proposed to explain the negative regulation of the hypothetical Lup permease involving a Lup1/Bul1 and Rsp5 complex that recognises and ubiquitinates the Lup permease resulting in its endocytosis and degradation in the presence of ammonium, is the simplest model to fit existing evidence. Alternatively, given that

Rsp5 also regulates cytoplasmic proteins (de la Fuente et al., 1997; Huibregtse et al., 1997), the Lup1/Bul1 and Rsp5 complex may be involved indirectly in regulation of the Lup permease, by negatively regulating a positive regulator of the Lup permease in the presence of nitrogen. Lup1/Bul1 may have a role in the ubiquitin pathway, distinct from substrate recognition. One could imagine that rapid transthiolation and transfer of ubiquitin by components of the ubiquitin pathway would be more efficient if these components either form a stable or transient multiprotein complex. Although this ubiquitin complex is hypothetical, Lup1/Bul1 and Rsp5 were shown to be associated with large complexes (Yashiroda et al., 1996) and the hect-domains of several E3 proteins have been shown to interact with specific, and in some cases multiple, E2 enzymes (Medintz et al., 1998). Binding by Lup1/Bul1 to Rsp5 therefore may not necessarily imply that both function together as an E3 complex. The structure of the ubiquitin system is hierarchal, with only two identified E1 enzymes carrying out all ubiquitin activation, whereas at least 13 E2 enzymes have been identified. However, as Lup1/Bul1 has an even narrower function than the E3 enzyme, Rsp5, Lup1/Bul1 is not predicted to function as an E1 or E2 enzyme. Moreover, members of E1 and E2 proteins have many sequence similarities, such as the ubiquitin conjugating (UBC) domain in E2 enzymes, and Lup1/Bul1 does not share any of these similarities. Yashiroda et al. (1998) have used a fluorescent dye, Lucifer Yellow, to test if *lup1/bul1 bul2* mutants are defective in endocytosis. Dye accumulated in the vacuole at restrictive temperatures. Therefore, Lup1/Bul1 also does not play a general role in endocytosis.

Unlike Lup1/Bul1, Rsp5 is essential for growth as various disruptions of *RSP5* result in cell inviability at all temperatures (Yashiroda et al., 1996). Thus, Lup1/Bul1 is predicted to be involved in the regulation of some, but not all, Rsp5 substrates. The Fur4 uracil permease is degraded following Rsp5-mediated ubiquitination at the approach of stationary phase, when protein synthesis is inhibited, and at a basal level during normal growth (Galan et al., 1996). If Lup1/Bul1 is also required for ubiquitination of Fur4, prevention of Fur4 degradation by *LUP1/BUL1* mutation may confer a growth advantage in conditions of uracil limitation in uracil prototrophic strains. However, growth of *LUP1/BUL1* and *lup1/bul1* strains, auxotrophic for uracil, was indistinguishable when grown on plates containing a uracil concentration gradient (Heinemann et al., 1994). These researchers have also reported that another Rsp5 substrate, Gap1, remains regulated in response to nitrogen source in *lup1/bul1* mutants.

This is not surprising as Gap1 function is regulated at the level of production by NCR, in addition to NCI, so total deregulation of Gap1 in ammonium environments would require disruption of both of these systems. Although expression of *GAP1* is strongly reduced in the presence of ammonium, a small amount is still produced (Jauniaux and Grenson 1990) which will be targeted for degradation by NCI. Also, NCI is involved in turnover of basal levels of Gap1 in the presence of proline (Springael and André, 1998). Thus, if Lup1/Bul1 is required with Rsp5 in the ubiquitination of Gap1, we would predict that Gap1 activity would be increased in the *lup1/bul1* mutant in the presence of ammonium and proline. Transport of [^{14}C]citrulline was indeed increased to varying degrees in the *lup1/bul1* mutant in both of these environments (Heinemann et al., 1994). In the presence of ammonium, the *lup1/bul1* strain displayed a slightly higher transport velocity of 325 pM per 10^7 cells per 15 min into a *lup1/bul1* strain, compared with 254 pM per 10^7 cells per 15 min into the wildtype. In the presence of proline, the difference was more obvious with the wildtype and *lup1/bul1* strains transporting [^{14}C]citrulline at velocities of 5266 and 17502 pM per 10^7 cells per 15 min, respectively (Heinemann et al., 1994). Whether or not Gap1 inactivation by NCI also requires Lup1/Bul1 could be determined by comparing transport in the presence of ammonium into *LUP1/BUL1* and *lup1/bul1* strains deficient in NCR. Alternatively, decreases in Gap1 transport, following addition of ammonium, to proline-grown *LUP1/BUL1* and *lup1/bul1* cells could be monitored in these strains.

The demonstration that Lup1/Bul1 is involved in the ubiquitin pathway provides an explanation for the apparent anomaly whereby Lup$^+$ variants were more sensitive than their Lup$^-$ counterparts to toxic analogs of hydrophobic and nonhydrophobic amino acid analogs. Entry of amino acid analogs into the yeast cell is followed by incorporation into proteins in the place of natural amino acids, resulting in misfolded proteins. Stress conditions, including those that generate aberrant proteins, elicit various cellular stress responses, such as induced expression of genes encoding a group of proteins that repair molecular damage and protect the cell from the detrimental effects of that stress. The genes encoding components of the ubiquitin pathway *UBC4, UBC5* (Seufert and Jentsch, 1990) and *UBI4* (encodes ubiquitin) (Finley et al., 1987) are examples of such stress-induced proteins. The ubiquitin pathway is responsible for the recognition and removal of aberrant proteins, thus disruption of components involved in this recognition and removal will result in accumulation of aberrant proteins and consequently, growth

inhibition. Indeed, sensitivity to toxic amino acid analogs has been a common method used for the isolation of ubiquitin pathway machinery. Like the *lup1/bul1* mutant, mutations in several components which may be involved in Lup1/Bul1 regulation of the Lup permease also result in canavanine sensitivity. These include the *rsp5-101* mutation (Yashiroda et al., 1996), disruption of *DOA4* (Hicke, 1997), *UBI4* (Finley et al., 1987), *UBC4* and *UBC5* (Seufert and Jentsch, 1990). That *lup1/bul1* variants are more sensitive than their wildtype counterparts to not only toxic amino acid analogs, but also the stress response-inducing conditions of high temperatures (Yashiroda et al., 1996), high salt concentrations (Yashiroda et al., 1998) and high cadmium levels indicates that Lup1/Bul1 may be a component of this stress response.

4.5 WHAT IS THE IDENTITY OF THE LUP PERMEASE?

This study has provided few significant insights as to the identity of the Lup permease. The virtually identical activity of the permease in *lup1/bul1* mutants in the presence of ammonium and proline nitrogen sources (Heinemann et al., 1994) suggests that the permease is only regulated by one, Lup1/Bul1-dependent, method of nitrogen regulation. Results from this study implicate that this nitrogen regulation mechanism comprises the post-translational NCI control system, hence the Lup permease is not predicted to be regulated at the transcriptional level by NCR. The Agp1 permease, although sharing a similar substrate specificity to the Lup permease (Iraqui et al., 1999a), is strongly negatively regulated by NCR (Schreve et al., 1998), therefore does not comprise the Lup permease. The Bap2 permease shares a high affinity for leucine with the Lup permease and the S2 permease has similar substrate specificity. Both are regulated negatively by ammonium, unlike the other permeases, Tat1, Tat2, Mup1 and Mup3, that transport leucine and other hydrophobic amino acids. Moreover, *BAP2* transcription is not regulated by NCR (Didion et al., 1996), but probably is regulated by NCI. Bap2 contains a conserved C-terminal motif required for ubiquitination and subsequent degradation of permeases sensitive to NCI regulation, the deletion of which results in increased transport by Bap2 (Grauslund et al., 1995). It is therefore possible that the Lup permease is actually Bap2 or S2, although Bap2 has different substrate specificity and S2 has different substrate affinity. A comparison of leucine transport in the presence of ammonium as a nitrogen source into *LUP1/BUL1* and *lup1/bul1* strains,

also containing various deletions of hydrophobic amino acid uptake permeases, should reveal which permease the Lup permease comprises, or indeed, whether it is unique.

4.6 HIGH MUTATION RATE TO LUP$^+$ PHENOTYPE

Mutation to the Lup$^+$ phenotype purportedly arises at a high frequency. The mutation frequency was approximately 4 x 10^{-6} after two to four days of growth, and rose to as high as 3 x 10^{-2} after 12 days of growth. This may not constitute the true mutation rate, however, as no effort was made to eliminate the possibility that continued cell growth and DNA metabolism of wildtype cells was still occurring (Heinemann et al., 1994). Wolfe et al. (1999) have also commented that resistance to the growth-inhibitory effects of isoflurane which may be conferred by mutations in *ZZZ1/LUP1/BUL1*, arises at a fairly high frequency. Although mutation frequencies were not reported, mutants were typically isolated from plates inoculated with 10^5 cells. The chance of a spontaneous mutation occurring at a particular nucleotide residue is estimated at approximately 10^{-9} to 10^{-10} per round of replication per base pair (Mathews and van Holde, 1990). Given that *LUP1/BUL1* is an approximately 3 kb gene, in one round of replication, one in every 10^6 to 10^7 cells would be predicted to have undergone a spontaneous change in the *LUP1/BUL1* sequence, although a proportion of these mutations would be silent. The high mutation rates reported therefore make it seem unlikely that mutation is occurring by spontaneous point mutation. Alternatively, *LUP1/BUL1* may contain a hotspot for transposon insertion, or recombination. However, determination of the nature of changes within *LUP1/BUL1* that result in two spontaneous mutants exhibiting the Lup$^+$ phenotype, has revealed that at least in these cases, point mutation, occurring at different regions of *LUP1/BUL1*, could explain the mutations.

The continued arisal of Lup$^+$ mutants on media that advantages the *lup1/bul1* mutant, past the window of time that mutants would be expected to occur if mutation had occurred at the time of plating, is reminiscent of reversions of *trp* operon missense mutations in *E. coli* (Hall, 1990), and Lys$^+$ revertants in *S. cerevisiae* (Steel and Jinks-Robertson, 1992), following plating on selective media. In both cases, continued cell division was not detected (although DNA turnover was not adequately excluded), thus late revertants may not have been due to cryptic growth of some cells. Moreover,

mutants were not found to accumulate under nonselective conditions. These results question the central tenet of evolutionary biology, that mutations arise randomly, rather than in response to a specific directive from the environment. Claims of selection-induced, 'Cairnsian' or directed mutation evoke controversy, however, and critics suggest that studies reporting these claims have been carried out without adequate controls (Lenski and Mittler, 1993; MacPhee, 1993).

That higher than predicted levels of spontaneous mutation to the Lup^+ phenotype are apparently occurring when cells are plated on media that starves the wildtype, is consistent with claims that the point mutation rate is increased under starvation conditions. DNA metabolism continues and chromosomal nicks and gaps accumulate under stress conditions, such as is encountered during stationary phase, and DNA errors may outpace DNA repair. Harris et al. (1997) have provided evidence that this may indeed occur in *E. coli*. Mismatch repair comprises the main mechanism for repair of errors that occur during DNA replication in *E. coli*. Levels of the mismatch repair proteins MutS and MutH declined with the replication rate during stationary phase, but still maintained a proper ratio of repair proteins to replication errors. However, a critical mismatch protein, MutL, did become limiting. Whether or not the Lup^+ phenotype is acquired at a higher rate in starvation conditions than nonstarvation conditions, or in selective rather than nonselective conditions, therefore remains to be determined.

Although both spontaneously arising Lup^+ mutants studied had mutations in the *LUP1/BUL1* gene, this does not necessarily imply that all Lup^+ mutants are *lup1/bul1* mutants. The involvement of Lup1/Bul1 in the ubiquitin pathway offers an alternative explanation to why mutation resulting in deregulation of the Lup permease and acquisition of isoflurane resistance appears to arise at a high frequency. As the ubiquitin pathway is a multiprotein cascade, mutation of any one of the components in this cascade would be predicted to result in the Lup^+ phenotype and isoflurane resistance. Moreover, only seven of 15 spontaneous isoflurane mutants had mutations in *ZZZ1/LUP1/BUL1* (Wolfe et al., 1999). Other genes isolated that, when mutated, displayed isoflurane resistance included *RSP5*, *ZZZ4/DOA1/UFD3*, *PRE1* and *PRE2*. Other proteins which may possibly give rise to the Lup^+ phenotype, when mutated, include the Lup or other leucine permeases, if mutated at regions required for their

negative regulation, and other negative regulators of leucine permeases. However, we have seen that the Lup^+ and Fpa^S phenotypes of nine individually isolated Lup^+ mutants could be complemented by the introduction of pJO13 (data not shown). These findings suggest that in each case, mutation was occurring in the *LUP1/BUL1* gene, although complementation of these phenotypes by *LUP1/BUL1* is not *prima facie* evidence that they have a mutation in the same, *LUP1/BUL1* gene.

The purportedly high mutation rate to the Lup^+ phenotype may have implications for the emergence of drug resistance that has diminished the efficacy of most therapeutic agents. Resistance of tumour cells to chemotherapy and pathogenic microorganisms to antimicrobial agents is often mediated by efflux pumps, which effectively decrease intracellular drug concentration. Acquisition of efflux-mediated drug resistance is considered to occur by a change in the specificity or regulation of an existing pump. Changes in pump specificity are conceivably rare events, as they require fortuitous mutation within the pump substrate-binding domain. Multidrug effluxes are also negatively regulated by the multicomponent ubiquitin pathway (Table 4.1). As mutations that result in deregulation of the Lup permease, an efflux pump in reverse, appear to occur at a high frequency, changes in regulation of pumps via the ubiquitin pathway may represent an important mechanism for acquisition of drug resistance.

4.7 CONCLUSIONS

The propensity of Lup^+ variants to better accumulate hydrophobic amino acids and their toxic analogs has been instrumental for developing a positive selection for strains containing the *LUP1* allele, for use in screening libraries for complementation of the *lup1* allele. As predicted, *lup1* variants were shown to be more sensitive than their progenitor to the hydrophobic amino acid analogs *m*-fluoro-D,L-phenylalanine, L-ethionine and L-methionine sulfoximine. Lup^+ strains were also unexpectedly more sensitive to the nonhydrophobic analogs L-canavanine and L-azaserine, raising questions as to the mechanism for this increased sensitivity. Is increased sensitivity due to elevated transport of these compounds by the permease/permeases normally regulated by Lup1, or is another mechanism involved?

This study has provided compelling evidence that *LUP1* is allelic to the *BUL1* gene. First, *BUL1*, when provided on low or high copy number plasmids, could complement the Fpa^S, Lup^+ and temperature sensitive phenotypes of *lup1* mutants. Temperature sensitivity is also a phenotype reported as characteristic of *bul1* mutants. Replacement of the chromosomal *BUL1* allele with *bul1::URA3* resulted in a strain portraying phenotypes identical to those observed for *lup1* mutants. In addition, the mutant Lup^+ and Fpa^S phenotypes of *lup1* and *bul1* mutants were not complemented when a *lup1* mutant was mated with a *bul1* mutant. Moreover, all offspring resulting from sporulation of this diploid were Lup^+ and Fpa^S suggesting that *LUP1* and *BUL1* occupied the same, or an extremely close, chromosomal position.

Identification of the *LUP1* locus has enabled the nature of mutation occurring within two spontaneously arising Lup^+ mutants to be determined. In both cases, the mutant phenotype was the result of a single base pair change within *LUP1/BUL1*. Establishment of the nature of the change within Lup^+ mutants has failed to provide us with a basis for a hypothesis on the extraordinary mutation rate of this locus, if this mutation rate is indeed high. More rigorous tests determining the true mutation rate of this locus are required, such as eliminating the possibility that continued DNA metabolism and growth of wildtype cells occurs on selective plates, and whether the same locus is involved each time the Lup^+ phenotype arises.

The finding that Lup1 is identical to Bul1, a protein that binds to Rsp5 and functions in substrate recognition in the ubiquitin pathway, has allowed us to propose a model for the negative regulation by Lup1/Bul1 of the Lup permease in the presence of ammonium. When this nitrogen source is present, a Lup1/Bul1-Rsp5 complex recognises the Lup permease and ubiquitinates it, which subsequently acts as a trigger for the internalization and degradation of this protein. In the presence of proline or in a *lup1/bul1* mutant, substrate recognition and/or ubiquitination no longer occurs, thus the permease remains active, and hydrophobic amino acid transport is allowed to continue. The observation that Lup1/Bul1 was unable to complement *lup1/bul1* phenotypes when it contained an altered PPxY motif, essential for binding of Lup1/Bul1 and Rsp5, is consistent with the model that Rsp5 function and binding to Lup1/Bul1 are essential for Lup permease regulation. More definitive proof of this theory awaits identification of the Lup permease.

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APPENDICES

APPENDIX 1: MEDIA

Unless specified otherwise, all media and media supplements were prepared in dH_2O, and sterilised by autoclaving for 15 to 20 min at 120°C and 1 atmosphere (15 pounds per square inch) pressure on liquid cycle.

Supplements For Yeast Minimal Media

Amino Acid/Supplement	*Concentration in Media (mg L^{-1})*
L-Leucine	80
Adenine	20
L-Lysine	60
L-Arginine	20
L-Tryptophan	20
L-Tyrosine	30
L-Threonine	200
L-Methionine	20
L-Phenylalanine	50
L-Histidine	20
Uracil	20

Antibiotic Concentrations Added To Media

Antibiotic	*Concentration in Media (μg mL^{-1})*
Ampicillin (Ap)	100
Kanamycin (Km)	50
Streptomycin (Sm)	50
Tetracycline (Tc)	25

All antibiotic stock solutions were filter sterilised.

Yeast Extract Peptone Dextrose (YPD)

Ingredient	*Concentration (L^{-1})*
Peptone	20 g
Yeast Extract	10 g
50% (w/v) Glucose (added after autoclaving)	40 mL

Synthetic Dextrose (SD)

Ingredient	*Concentration (L^{-1})*
Gibco BRL Yeast Nitrogen Base without amino acids, without ammonium sulphate	1.43 g
Ammonium sulphate	5 g
50% (w/v) Glucose (added after autoclaving)	40 mL

Luria Bertani (LB)

Ingredient	*Concentration (g L^{-1})*
Bactotryptone	10
Yeast Extract	5
NaCl	5
pH 7.0-7.4	

McClary's Medium

Ingredient	*Concentration (L^{-1})*
Potassium acetate	10 g
Yeast Extract	2.5 g
50% (w/v) Glucose (added after autoclaving)	2 mL

LBMM Medium

Ingredient	*Concentration*
LB	
Maltose	0.2% (w/v)
Magnesium sulphate	10 mM

Filter sterilised maltose and $MgSO_4$ stock solutions were added to LB medium following autoclaving.

SOB Medium

Ingredient	*Concentration (g L^{-1})*
bacto-tryptone	20
bacto-yeast extract	5
Sodium chloride	0.584
Potassium chloride	0.186

SOC Medium

Ingredient	*Concentration (100 mL^{-1})*
SOB	98 mL
2 M Magnesium stock solution	1 mL
2 M Glucose solution	1 mL

Magnesium (filter sterilised) and Glucose stock solutions were added following autoclaving of SOB.

APPENDIX 2: BUFFERS AND SOLUTIONS

All buffers and solutions were prepared in dH_2O.

COMMON BUFFERS AND SOLUTIONS

20 × SSC

Ingredient	*Concentration (M)*
Sodium chloride	3
Sodium citrate dihydrate	0.3
pH 7.0	

$T_{10}E_1$ Buffer

Ingredient	*Concentration (mM)*
Tris-HCl	10
EDTA	1
pH 8.0	
autoclaved	

Bromophenol Blue Loading Buffer

Ingredient	*Concentration*
glycerol	80 % (v/v)
EDTA	20 mM
bromophenyl blue	0.025 % (w/v)

10 × Tris-Borate Electrophoresis Buffer (TBE)

Ingredient	*Concentration (L^{-1})*
Tris-HCl	108 g
boric acid	55 g
0.5 M EDTA, pH 8.0	40 mL

ALKALINE LYSIS AND LITHIUM CHLORIDE PLASMID PREPARATIONS FROM *E. COLI*

Solution 1

Ingredient	*Concentration (mM)*
glucose	50
Tris-HCl (pH 8.0)	25
EDTA (pH 8.0)	10

autoclaved

Solution 2

Ingredient	*Concentration*
NaOH	0.2 M
SDS	1% (w/v)

Solution 2 was prepared fresh before each use.

Solution 3

Ingredient	*Concentration*
sodium acetate	3 M

RNase (free of DNase)

Ingredient	*Concentration*
RNA	10 mg mL^{-1}
Sodium acetate	0.01 M

pH 5.2

The solution was heated at 100°C for 15 min, and allowed to cool slowly at RT.

LITHIUM ACETATE-MEDIATED YEAST TRANSFORMATION SOLUTIONS

Single-Stranded Carrier DNA

2 mg high molecular weight Herring sperm DNA (Gibco BRL) was dissolved in1 mL TE buffer. Prior to use, an aliquot was boiled for 5 min and cooled in an ice slurry. DNA was stored at -20°C.

Lithium Acetate

Ingredient	*Concentration*
Lithium acetate	1.0 M
pH 8.4-8.9	
sterilised by autoclaving	

Polyethylene Glycol (PEG)

Ingredient	*Concentration*
polyethylene glycol, MW 3350	50 (w/v)
sterilised by autoclaving	

PLASMID EXTRACTION FROM YEAST

Buffer A

Ingredient	*Concentration*
Triton X-100	2 % (v/v)
SDS	1 % (w/v)
NaCl	100 mM
Tris-HCl, pH 8.0	10 mM
EDTA, pH 8.0	1 mM

YEAST CHROMOSOMAL DNA PREPARATION SOLUTIONS

SCEM (made fresh)

Ingredient	*Concentration*
sorbitol	1 M
EDTA, pH 8	60 mM
sodium citrate	0.1 M
β-mercaptoethanol	1% (v/v)
yeast lytic enzyme	1 mg mL^{-1}
pH 7.0	

STE

Ingredient	*Concentration*
SDS	0.5% (w/v)
Tris, pH 9.7	100 mM
EDTA	50 mM

Potassium Acetate, 2.55 M

Ingredient	*Concentration (100 mL^{-1})*
Potassium acetate	25.03 g
Glacial acetic acid	11.5 mL
pH 4.8	

E. COLI ELECTROPORATION SOLUTIONS

2 M Magnesium Stock Solution

Ingredient	*Concentration (100 mL^{-1})*
$MgCl_2$.$6H_2O$	20.33 g
$MgSO_4$.$7H_2O$	24.65 g
filter sterilised	

WB

Ingredient	*Concentration*
Ultra-pure glycerol	10% (v/v)
autoclaved	

VACUUM TRANSFER SOLUTIONS

Depurination Solution (0.2M HCl)

Ingredient	*Concentration (L^{-1})*
conc. HCl	17.2 mL

Neutralisation Solution

Ingredient	*Concentration (M)*
Tris-HCl	0.5
NaCl	3.0
pH 7.0	

Denaturation Solution

Ingredient	*Concentration (M)*
NaCl	1.5
NaOH	0.5

SOUTHERN BLOTTING SOLUTIONS

50 × Denhardt's Solution

Ingredient	*Concentration (g L^{-1})*
Ficoll, MW 400 000	10
Polyvinylpyrrolidone	10
Bovine serum albumin	10

Prehybridisation Buffer

Ingredient	*Concentration (100 mL^{-1})*
SSC	6 ×
SDS	0.5% (w/v)
50 × Denhardt's solution	10 mL
100 μg mL^{-1} denatured herring sperm DNA	1 mL

Hybridisation Buffer

Ingredient	*Concentration (100 mL^{-1})*
SSC	6 ×
SDS	0.5% (w/v)
100 μg mL^{-1} denatured herring sperm DNA	1 mL

STS

Ingredient	*Concentration*
SSC	0.1 ×
Tris-HCl, pH 8	0.2 M
SDS	0.5 % (w/v)

SOLUTIONS FOR SEPARATION OF SPORES FROM VEGETATIVE CELLS

Pretreatment Solution

Ingredient	*Concentration (M)*
EDTA	0.02
Tris, pH 7.8	0.02
β-mercaptoethanol	0.1
sorbitol	1.2

Protoplasting Solution

Equal volumes of 0.016 M sodium citrate and 0.08 M potassium dihydrogen phosphate (pH 5.8) were mixed

SEQUENCING SOLUTIONS AND PARAMETERS

4% Long Ranger™ Gel Preparation

Ingredient	*Concentration (75 mL^{-1})*
Urea	31.5 g
Long Ranger™ Gel Solution	6.0 mL
10 × TBE*	9.0 mL
dH_2O	36 mL
Fresh 10% (w/v) APS	500 µL
TEMED	50 µL

10 × TBE*

Ingredient	*Concentration (mM)*
Tris Base	890
Boric Acid	890
EDTA	20

Running Conditions

Data collection configuration file	66 cm STD col
Quick Sequence IR configuration file Quik	66 cm.col
Voltage	2000 V
Current	25.0 mA
Power	45.0 watts
Temperature	45°C
Scan speed	2 (1.2 frames $hour^{-1}$)
Prerun time	30 min
Frames to collect	32

APPENDIX 3: PCR PRIMERS

The starting ATG of *BUL1* and *BUL2* ORFs was defined as +1.

JO1, *BUL1* forward (-100 to -81):

5'-TAT TGC AAT GAA GAG CCG CA-3'

JO2, *BUL1* reverse (+680 to +699):

5'-GTC ATC AAG TAT GGG AGG TA-3'

JO3, *BUL1* forward (+680 to +699) and M13 19-mer forward sequence:

5'-CAC GAC GTT GTA AAA CGA CTA CCT CCC ATA CTT GAT CAC-3'

JO4, *BUL1* reverse (+1470 to +1489) and M13 reverse sequence:

5'-GGA TAA CAA TTT CAC ACA GGT TCT TTC TCC GAC GAC ATT G-3'

JO5, *BUL1* forward (+1470 to 1489):

5'-CAA TGT CGT CGG AGA AAG AA-3'

JO6, *BUL1* reverse (+2181 to +2200):

5'-GAA TTT CTG GCG GAT CAT GA-3'

JO7, *BUL1* forward (+2181 to +2200):

5'-TCA TGA TCC GCC AGA AAT TC-3'

JO8, *BUL1* reverse (+3010 to + 3029):

5'-GGA ATG CAA AGG ACG CGA TA-3'

JO9, *BUL1* reverse (+3874 to +3893):

5'-GTC ATG GAG CCT TCC TGA AA-3'

JO11, *BUL2* forward (-1200 to -1181):

5'-TGT TGC ACA GGA TGG AAG CA-3'

JO12, *BUL2* reverse (+3245 to +3264):

5'-GGG CTC GAT GAT TGC TAA TG-3'

M13/PUC 19-mer IR sequencing forward primer:

5'-IR-CACGACGTT GTA AAA CGA A-3'

APPENDIX 4: RESTRICTION SITES IN COMPLEMENTING PLASMIDS

RESTRICTION SITES WITHIN PJO21

Sites for cleavage by selected restriction enzymes within the insert of pJO21, as illustrated in Figure 3.5, are listed below. Exact location of sites was determined from sequence analysis of chromosome II surrounding the *ARO4* and *HIS7* genes, obtained from the National Centre for Biotechnology Information. Initial analyses of restriction enzyme digestion revealed that the first digestion site for *Hin*dIII (on left hand side of pJO21 map) was approximately 200 bp into the insert. Cleavage by *Hin*dIII occurs after the first A in the recognition sequence AAGCTT. This base was therefore arbitrarily designated as nucleotide number 200. Nucleotide numbering continues from left to right. The *HIS7* and *ARO4* coding regions lie at nucleotides 3725 to 2070 and 5254 to 4145, respectively.

Enzyme	*Cleavage Site (nucleotide number)*		
*Bam*HI	3310		
*Bgl*II	2658		
*Eco*RI	2133	3262	3349
*Eco*RV	362	3827	
*Hind*III	200	1388	7374
*Pvu*II	655	5996	
*Sal*I	2514	3552	

RESTRICTION SITES OF PJO13

The positions where selected restriction enzymes cleave the insert within pJO13, as depicted in Figure 3.4, are listed below. Following manual restriction digestion analyses, exact cleavage sites were determined from analysis of DNA sequence from *S. cerevisiae* chromosome XIII, obtained from the National Centre for Biotechnology

Information. The initial *Cla*I site (farthest left site in Figure 3.4.) within pJO13 was determined by restriction digestion analyses to be 1300 bp from the beginning of the insert. *Cla*I cleaves after the first T in the recognition site ATCGAT, thus this nucleotide was designated as number 1300. The *BUL1/LUP1* coding region occurs at nucleotides 10470 to 7543.

Enzyme	*Cleavage Site (nucleotide number)*				
*Bam*HI	10248				
*Bgl*II	5005	6025			
*Cla*I	1300	1339	8265		
*Eco*RI	6178	6781			
*Eco*RV	7572	8001	8958	10101	
*Hind*III	3437	3505	3559	3992	8157
*Pvu*II	2333	8013	8967	10998	11538
*Sac*I	4263	6813			
*Sal*I	2659				

APPENDIX 5: GLOSSARY OF GENES RELEVANT TO STUDY

Gene	Gene Product Function/Description
AAP1	"Amino acid permease factor". Required for processing and translocation of amino acid permeases from endoplasmic reticulum to membrane. Allelic to *APF1* and *SHR3*.
AAT1	"Amino acid transport". Mutants are unable to grow on rich media when auxotrophic for leucine, thus may positively regulate leucine permease/s.
ACT1	"Actin". Involved in cell polarisation, endocytosis, and other cytoskeletal functions.
ADE6	"Adenine". 5'-phosphoribosylformyl glycinamidine synthetase, catalyses fourth step in *de novo* purine biosynthesis.
AGP1	"Asparagine and glutamine permease". Broad specificity amino acid permease. Allelic to *WAP1*.
APF1	Required for processing and translocation of amino acid permeases from endoplasmic reticulum to membrane. Allelic to *AAP1* and *SHR3*.
AREA	GATA zinc finger nitrogen regulatory factor in *Aspergillus nidulans*.
ARO4	Tyrosine-regulated DAHP synthase involved in Shikimate pathway for the biosynthesis of aromatic amino acids.
AUA1	"Amino acid uptake activation". Protein involved in ammonia regulation of Gap1.
BAP1	"Branched-chain amino acid permease". Zinc finger protein rather than permease, involved in positive regulation of amino acid-inducible expression of various permease genes and pre-tRNA splicing of specific tRNA species. Allelic to *STP1*.
BAP2	"Branched-chain amino acid permease". Branched chain amino acid permease specific for leucine, valine, and isoleucine
BAP3	"Branched-chain amino acid permease". Allelic to *PAP1*.
BUL1	"Binds ubiquitin ligase". Protein involved in the ubiquitination pathway and regulates putative Lup amino acid permease in the presence of ammonium. Allelic to *LUP1*, *DAG1*, *RDS1* and *ZZZ1*.
BUL2	"Binds ubiquitin ligase". Protein with similarity to Bul1.

Gene	Gene Product Function/Description
CAN1	"Canavanine resistance". Permease for basic amino acids including arginine, lysine, and histidine.
CIM3	ATPase component of 26S proteasome complex.
CIM5	ATPase component of the 26S proteasome complex
DAG1	"*DSK2*-adjacent gene". Protein involved in the ubiquitination pathway and regulates putative Lup amino acid permease in the presence of ammonium. Allelic to *LUP1*, *BUL1*, *RDS1* and *ZZZ1*.
DAL4	Allantoin permease.
DAL5	Allantoate and ureidosuccinate permease. Allelic to *UEP*.
DAL80	GATA-type zinc finger transcriptional represser involved in negative regulation of NCR-sensitive gene expression. Allelic to *DEH1* and *GZF1*.
DAL81	Cyc6-Zn^{2+}-type transcription activator required for function of some nitrogen catabolic pathways. Allelic to *UGA35* and *DURL*.
DEH1	"Dal eighty homolog protein". GATA-type zinc finger transcriptional represser involved in negative regulation of NCR-sensitive gene expression. Allelic to *NIL2* and *GZF1*.
deoC	*E. coli* gene encoding a component involved in deoxyribose synthesis.
deoR	*E. coli* regulatory gene mutation allowing constitutive expression of genes for deoxyribose synthesis.
DIP5	High affinity, high capacity "dicarboxylic amino acid permease".
DOA1	"Degradation of alpha". Ubiquitin C-terminal hydrolase (E4 enzyme) involved in recycling of ubiquitin from protein substrates. Allelic to *ZZZ4* and *UFD3*.
DOA4	"Degradation of alpha". Ubiquitin C-terminal hydrolase (E4 enzyme) involved in recycling of ubiquitin from protein substrates. Allelic to *NPI2* and *MUT4*.
DURL	Cyc6-Zn^{2+}-type transcription activator required for function of some nitrogen catabolic pathways. Allelic to *DAL81* and *UGA35*.
end1	*E. coli end1* mutants have abolished nonspecific endonuclease activity.
END3	"Endocytosis". Protein required for endocytosis of various plasma membrane proteins.

Gene	Gene Product Function/Description
END4	"Endocytosis". Required for endocytosis of various plasma membrane proteins.
FUR4	"Fluorouracil resistance". Uracil permease.
GAL2	"Galactose permease".
GAP1	"General amino acid permease". Wide specificity amino acid permease that transports all naturally occurring amino acids.
GAT1	GATA zinc finger transcription factor involved in positive regulation of NCR-sensitive gene expression.
GCN4	"General control nonderepressible". Transcriptional activator of various genes for amino acid biosynthesis, regulates general control in response to amino acid or purine starvation,
GDHA/GDH1	NADP-linked glutamate dehydrogenase which combines ammonia and α-ketoglutarate to form glutamate.
GDH2	NAD-linked glutamate dehydrogenase, primary pathway for generating ammonia from glutamate
GDHCR	Negative regulator of nitrogen catabolite repression; acts by inhibition of Gln3 in the presence of preferred nitrogen sources. Allelic to *URE2* and *USU.*
GLN1	Glutamine synthase which combines ammonia to glutamate in ATP-driven reaction reaction.
GLN3	Positive, GATA-type zinc finger transcriptional regulator of NCR-sensitive gene expression.
GNP1	High affinity "Glutamine permease".
GPA2	Guanine nucleotide-binding protein α-subunit involved in regulation of the cAMP pathway.
GRR1	"Glucose repression resistant". F-box protein of the SCF^{Grr1} ubiquitin ligase complex, that targets a number of proteins for degradation, also required for glucose repression and for glucose and cation transport.
GZF3	"GATA zinc finger factor". GATA-type zinc finger transcriptional represser involved in negative regulation of NCR-sensitive gene expression. Allelic to *DEH1* and *DAL80.*
HIP1	"Histidine permease".

Gene	Gene Product Function/Description
HIS3	"Histidine". Imidazoleglycerolphosphate (IGP) dehydratase, catalyses seventh step in histidine biosynthesis pathway.
hsdR	*E. coli* gene encoding one subunit of the restriction enzyme *Eco*K responsible for cutting DNA. *hsdR* strains methylate but do not cut DNA.
hsdM	*E. coli* gene that encodes the subunit of methylase that methylates *Eco*K restriction sites.
kan	Gene that confers "Kanamycin resistance".
lacY	*E. coli* gene encoding β–galactoside permease.
lacZ	*E. coli* gene encoding β-galactosidase.
LET1	Mutants have inactivated *BAP2* permease. Possibly allelic to *BAP2.*
LET2	Mutants have inhibited transport by S2 permease.
LEU2	Leucine biosynthetic enzyme, 3-isopropylmalate dehydrogenase.
LEU3	Transcription factor regulating genes of branched chain amino acid biosynthesis pathways, acts as both a represser and an inducer.
LRP1	"Leucine-responsive regulatory protein". *E. coli* protein involved in regulation of expression of genes in response to leucine levels.
LUP1	"Leucine uptake". Protein involved in the ubiquitination pathway and regulates putative Lup amino acid permease in the presence of ammonium. Allelic to *DAG1*, *BUL1*, *RDS1* and *ZZZ1*.
LYP1	High affinity "lysine permease".
MAL61	"Maltose permease".
mcrA,B,C	*E. coli mcrA*, *B* and *C* mutants have methyl cytosine restriction systems mutated.
MDM1	Intermediate filament protein involved in organelle inheritance and in the regulation of mitochondrial morphology.
MEP1	Ammonia permease of high capacity and moderate affinity.
MEP2	Ammonia permease of low capacity and high affinity.
MSC4	Upstream regulator of MAP kinase pathway in *Schizosaccharomyces pombe*.
MUP1	"Methionine uptake". High specificity methionine permease.

Gene	Gene Product Function/Description
MUP3	"Methionine uptake". Low affinity, wide specificity methionine permease which may function as an ammonium sensor.
MUT2	Ubiquitin-protein ligase involved in turnover of a number of proteins including NCI-regulated permeases. Allelic to *RSP5* and *NPI1*.
MUT4	Allelic to *NPI2* and *DOA4*.
MUTS,H,L	*E. coli* mismatch repair proteins.
NIL2	GATA-type zinc finger transcriptional represser involved in negative regulation of NCR-sensitive gene expression. Allelic to *DEH1* and *GZF1*.
NIT2	GATA-type zinc finger nitrogen regulatory transcription factor in *Neurospora crassa.*
NPI1	"Nitrogen permease inactivator". Ubiquitin-protein ligase involved in turnover of a number of proteins including NCI-regulated permeases. Allelic to *RSP5* and *MUT2*.
NPI2	"Nitrogen permease inactivator". Ubiquitin C-terminal hydrolase (E4 enzyme) involved in recycling of ubiquitin from protein substrates. Allelic to *DOA4* and *MUT4*.
NPR1	"Nitrogen permease reactivator". Putative serine-threonine protein kinase involved in regulating transport systems for nitrogen source nutrients.
NPR2	"Nitrogen permease regulator". Involved in positive, post-translational regulation of permeases in presence of urea and proline.
NUT1	GATA-factor involved in nitrogen regulation in *Magnaporte grisea.*
NRE	GATA-factor involved in nitrogen regulation in *Penicillium chrysogenum.*
PAP1	"Putative amino acid permease". Branched-chain amino acid permease. Allelic to *BAP3*.
PEP4	Aspartyl protease required for activation of various degradative enzymes.
PER1	Mutants are no longer able to inactivate Gap1 in the presence of ammonium.
pgr	A mutant allele of *GAP1* containing a glutamate to lysine substitution within the EEKAI sequence.

Gene	Gene Product Function/Description
ponA	*E. coli ponA* mutants are penicillin binding protein-deficient.
PRE1	Catalytic subunit of the proteasome.
PRE2	Proteasome subunit involved in chymotrypsin-like activity.
PTR2	Peptide permease nitrogen-repressible transporter of di- and tri-peptides.
PTR3	Involved in positive regulation of amino acid-inducible expression of various permease genes.
PUT1	"Proline utilisation gene". Proline oxidase which catalyses first step in synthesis of glutamate from proline.
PUT2	"Proline utilisation gene". Delta-1-pyrroline-5-carboxylate dehydrogenase which carries out the second step in proline degradation.
PUT4	"Proline utilisation gene". High affinity proline permease.
RAA1,2,3,4	Mutation confers "resistance to toxic amino acids". May be positive regulators of amino acid permeases.
RAS2	GTP-binding protein involved in regulation of cAMP pathway.
RDS1	"Respiration deficiency supressor". Protein involved in the ubiquitination pathway and regulates putative Lup amino acid permease in the presence of ammonium. Allelic to *DAG1*, *BUL1*, *LUP1* and *ZZZ1*.
recA	*E. coli* gene whose product is involved in recombination by promoting transfer of strands between DNA molecules.
relA	"Relaxed phenotype". Mutation of this *E. coli* gene allows RNA synthesis in the absence of protein synthesis.
RGT2	"Restores glucose transport". Glucose transporter and sensor responsible for induction of gene expression in the presence of high glucose.
RPB1	Largest subunit of RNA polymerase II.
rpsL	*E. coli* gene encoding a ribosomal protein. *rpsL* mutants are streptomycin resistant.
RSP5	Ubiquitin-protein ligase involved in turnover of a number of proteins including NCI-regulated permeases. Allelic to *MUT2* and *NPI1*.

Gene	Gene Product Function/Description
SHR3	"Super high histidine resistant". Required for processing and translocation of amino acid permeases from endoplasmic reticulum to membrane. Allelic to *AAP1* and *APF1*.
SNF3	"Sucrose nonfermenting". High-affinity glucose transporter and sensor responsible for induction of gene expression in the presence of low glucose.
SSY1	Putative amino acid sensor involved in positive regulation of amino acid-inducible expression of various permease genes.
SSY2	Zinc finger protein involved in positive regulation of amino acid-inducible expression of various permease genes and pre-tRNA splicing of specific tRNA species. Allelic to *BAP1* and *STP1*.
SSY3	May have a positive role in amino acid inducible expression of various permease genes.
SSY5	May have a positive role in amino acid inducible expression of various permease genes.
STE2	"Sterile". Pheromone α-factor receptor.
STE3	"Sterile". Pheromone a-factor receptor.
STE6	"Sterile". ABC transporter responsible for export of a-factor mating pheromone.
STP1	"Species-specific tRNA processing". Zinc finger protein involved in positive regulation of amino acid-inducible expression of various permease genes and pre-tRNA splicing of specific tRNA species. Allelic to *BAP1* and *SSY2*.
STS1	ABC-type drug-efflux pump involved in resistance to multiple drugs.
supO	*E. coli SupO* strains are non permissive for vectors carrying amber mutations.
supE	*E. coli* suppressor tRNA for amber mutations. Functions by inserting glutamine at UAG codons.
TAP42	Involved in TOR signalling pathway for activation of cell growth in response to nutrient availability.
TAT1	High-affinity tyrosine permease.
TAT2	High affinity tryptophan permease.

Gene	Gene Product Function/Description
TOR1	"Target of rapamycin". Kinase involved in TOR signalling pathway for activation of cell growth in response to nutrient availability.
TOR2	"Target of rapamycin". Similar kinase to Tor1, involved in TOR signalling pathway for activation of cell growth in response to nutrient availability.
TRP1	"Tryptophan". Phosphoribosylanthranilate isomerase, catalyzes the third step in the tryptophan biosynthesis pathway.
TYR1	"Tyrosine". Prephenate dehydrogenase, involved in tyrosine biosynthesis pathway.
UBA1	"Ubiquitin activating (E1) enzyme".
UBA2	"Ubiquitin activating (E1) enzyme".
UBC4	"Ubiquitin conjugating (E2) enzyme". Together with Ubc5, Ubc4 is responsible for the majority of ubiquitin-dependent protein degradation of short-lived and abnormal proteins.
UBC5	"Ubiquitin conjugating (E2) enzyme". Together with Ubc4, Ubc5 is responsible for the majority of ubiquitin-dependent protein degradation of short-lived and abnormal proteins.
UBI1	"Ubiquitin". Fusion protein comprised of ribosomal protein L40 (C-terminal half) and ubiquitin (N-terminal half).
UBI4	"Ubiquitin". Ubiquitin polyprotein from which mature ubiquitin is cleaved from.
UEP	Allantoate and ureidosuccinate permease. Allelic to *DAL5*.
UFD3	Ubiquitin C-terminal hydrolase (E4 enzyme) involved in recycling of ubiquitin. Allelic to *DOA1* and *ZZZ4*.
UGA1	4-Aminobutyrate aminotransferase (GABA transaminase) which is involved in production of gamma-aminobutyric acid.
UGA4	Amino acid permease with high specificity for 4-aminobutyric acid (GABA).
UGA35	Cyc6-Zn^{2+}-type transcription activator required for function of some nitrogen catabolic pathways. Allelic to *DAL81* and *DURL*.
URA3	"Uracil". Orotidine-5'-phosphate decarboxylase, catalyzes sixth step of pyrimidine biosynthesis pathway.

Gene	Gene Product Function/Description
URE2	Negative regulator of nitrogen catabolite repression; acts by inhibition of Gln3 in the presence of preferred nitrogen sources. Allelic to *GDHCR* and *USU*.
USU	Negative regulator of nitrogen catabolite repression; acts by inhibition of Gln3 in the presence of preferred nitrogen sources. Allelic to *URE2* and *GDHCR*.
WAP1	"Wide-specificity amino acid permease". Allelic to *AGP1*.
WEE1	Serine/threonine protein kinase in *Schizosaccharomyces pombe*.
ZRT1	Zinc permease.
ZZZ1	Protein involved in the ubiquitination pathway and regulates putative Lup amino acid permease in the presence of ammonium. Allelic to *DAG1*, *BUL1*, *LUP1* and *RDS1*.
ZZZ4	Ubiquitin C-terminal hydrolase (E4 enzyme) involved in recycling of ubiquitin. Allelic to *DOA1* and *UFD3*.